Orchidectomy increases expression and activity of Cu/Zn-superoxide dismutase, while decreasing endothelial nitric oxide bioavailability

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Abstract

This study examines the effect of male sex hormones on the release, metabolism and function of endothelial nitric oxide (eNO) in rat aorta. Aortic segments from orchidectomized and control male Sprague–Dawley rats were used to measure eNO synthase (eNOS) expression, nitric oxide (NO) release, acetylcholine (ACh)- and sodium nitroprusside (SNP)-induced relaxation and Cu/Zn-superoxide dismutase (SOD) expression and activity. eNOS expression as well as basal and ACh-induced NO release were similar in arteries from both groups of rats. Basal superoxide anion production was similar in arteries from both groups, while ACh-induced superoxide anion formation was greater in arteries from orchidectomized than control rats. Orchidectomy increased the vasodilator effect induced by ACh, but did not alter that induced by SNP. SOD, a superoxide

anion scavenger, did not modify the SNP-induced relaxation in aortas from control or orchidectomized rats. The membrane-permeable mimetic of SOD, tempol, increased the SNP-induced relaxation more in aortas from orchidectomized than control rats. The effect of endogenous SOD inhibitor, diethyl-dithiocarbamate, reduced the relaxation induced by SNP in segments from both groups of rats. The expression and activity of Cu/Zn-SOD were greater in aortas from orchidectomized than control rats. These data show that endogenous male sex hormone deprivation altered neither eNOS expression nor eNO release, while it increased the expression and activity of Cu/Zn-SOD. However, the predominant vascular effect of orchidectomy is to increase NO metabolism.

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Introduction

The role of androgens in vascular function is receiving considerable research interest (Sader & Celermajer 2002), since recent studies reported some protective actions of androgens in males (Liu *et al.* 2003, Perusquia 2003, Littleton-Kearney & Hurn 2004). Indeed, testosterone has been reported to have antiatherogenic actions (Larsen *et al.* 1993, Bruck *et al.* 1997, Alexandersen *et al.* 1999, Nathan *et al.* 2001) and improve myocardial ischaemia in men with coronary artery disease (Wu & Weng 1993, Rosano *et al.* 1999, English *et al.* 2002). One of the proposed beneficial factors is the interaction between androgens and endothelial nitric oxide (eNO; Tep-areenan *et al.* 2003, Jones *et al.* 2004).

The crucial role of eNO in vasomotor tone regulation has been widely recognized (Vanhoutte 1996, Busse & Fleming 2003). eNO is synthesized by eNO synthase (eNOS; Förstermann *et al.* 1991, Pollock *et al.* 1991, 1993), and its function is regulated by multiple stimuli.

Most of the studies have been focused on analysing the effects of androgenic derivatives on distinct aspects of the NO pathway. Indeed, increase (Simoncini *et al.* 2003), decrease (Chatrath *et al.* 2003) or no change (McNeill *et al.* 1999) of eNOS expression by androgenic derivatives have all

been described. Likewise, increase (Liu & Dillon 2002, Simoncini *et al.* 2003) or decrease (Mukherjee *et al.* 2001) of eNOS activity by androgens has both been reported. In addition, other investigations have shown that the eNO vasodilator effect is increased (Wynne & Khalil 2003, Orshal & Khalil 2004) or decreased (Ba *et al.* 2001, Gonzales *et al.* 2004) by androgens. Despite all these findings, there is a lack of experimental data on the specific effects of endogenous male sex hormone in those issues, when they are studied simultaneously.

The vascular effect of eNO is known to be determined by its bioavailability, and superoxide anion is an important contributor to any reduction of NO bioactivity. Therefore, the elimination of superoxide anion within vessel walls is fundamental and is performed by superoxide dismutases (SODs). In this context, it has been reported that the expression and/or activity of SODs are increased in pathological conditions like atherosclerosis (Kobayashi *et al.* 2002), in which the production of superoxide anions is increased (Wolin 2002). On the other hand, several studies have demonstrated androgen antioxidant properties (Békési *et al.* 2000, Yorek *et al.* 2002) and we observed an increased superoxide anion production in mesenteric artery from orchidectomized rats that had an increased neuronal NO

metabolism (Martín *et al.* 2005). In view of these data, we hypothesized that endogenous male sex hormones could influence the production of superoxide anion, as well as the expression and/or activity of SOD.

The aim of the present study, therefore, is to analyse whether endogenous male sex hormones influence the release, metabolism and/or function of eNO in rat aorta.

Material and Methods

Animal housing and protocols

Male Sprague–Dawley rats (6 months old) were used. These were divided into two groups: control and castrated males. All animals were housed in the Animal Facility of the Universidad Autónoma de Madrid (Registration number EX-021U) according to directives 609/86 CEE and R.D. 233/88 of the Ministerio de Agricultura, Pesca y Alimentación of Spain. Deprivation of male sex hormones was induced by gonadectomy at 7 weeks of age, and 4 months later the animals were killed. The observation of atrophy of the seminal vesicles confirmed successful surgery. Rats were killed by CO₂ inhalation; the aorta was carefully dissected out, cleaned of connective tissue and placed in Krebs–Henseleit solution (KHS) at 4 °C. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the USA National Institutes of Health (NIH publication no. 85-23 revised 1985, Bethesda, MD, USA).

Western blot analysis of eNOS and Cu/Zn-SOD expression

For Western blot analysis of eNOS protein expression, aortic segments were homogenized in a boiling buffer composed of 1mM sodium vanadate (a protease inhibitor), 1% SDS and 0.01M Tris–HCl (pH 7.4). Homogenates containing 16.5 µg protein were electrophoretically separated on a 7.5% SDS-PAGE for eNOS and on a 12% SDS-PAGE for Cu/Zn-SOD, then transferred to polyvinylidene difluoride membranes (Bio-Rad Immun-Blot overnight at 4 °C, 230mA, using a Bio-Rad Mini Protean III System; Bio-Rad Laboratories) containing 25mM Tris, 190mM glycine, 20% methanol and 0.05% SDS. Prestained SDS-PAGE broad-range standards (Bio-Rad Laboratories) were used as molecular mass markers. The membrane was blocked for 3h at room temperature in Tris-buffered saline solution 100mM, 0.9% (w/v) NaCl, 0.1% SDS with 5% non-fat powdered milk before being incubated overnight at 4 °C with mouse monoclonal antibody for eNOS (1:1000 dilution), purchased from Transduction Laboratories (Lexington, UK), or with rabbit polyclonal antibody for Cu/Zn-SOD (1:1000 dilution), purchased from StressGen Bioreagents (Victoria, BC, Canada). After washing, the membrane was incubated with a 1:1000 dilution of antimouse or antirabbit immunoglobulin G antibody respectively, conjugated to horseradish peroxidase (Amersham International Plc). The membrane was thoroughly washed and

the immunocomplexes were detected using an enhanced horseradish peroxidase/luminol chemiluminescence system (ECL Plus, Amersham International Plc) and subjected to autoradiography (Hyperfilm ECL, Amersham International Plc). Signals on the immunoblot were quantified using a computer program (NIH Image V1.56; NIH, Bethesda, MD, USA). The same membrane was used to determine α -actin expression, and the content of the latter was used to correct eNOS or Cu/Zn-SOD expression in each sample, by means of a monoclonal antibody anti- α -actin (1:2000 dilution, Sigma).

NO release

Aortic segments from control and orchidectomized rats were subjected to a resting tension of 1 g as indicated for the vascular reactivity. After an equilibration period of 60 min, arteries were incubated with the fluorescent probe 4,5-diaminofluorescein (DAF-2, 0.5 µM) for 45 min. Then, the medium was collected to measure the basal NO release. Once the organ bath was refilled, cumulative acetylcholine (ACh) concentrations (0.1 nM–10 µM) were applied. The fluorescence of the medium was measured at room temperature using a spectrofluorimeter (LS50 Perkin–Elmer Instruments, FL WINLAB Software, Bucks, UK) with excitation wavelength set at 495 nm and emission wavelength at 515 nm.

Blank measures were collected in the same way, but without aortic segments in order to subtract background emission. Some assays were performed in the presence of L-NAME (0.1 mM) to ensure the specificity of the method. The amount of NO released was expressed as arbitrary units per milligram tissue.

Superoxide anion production

Superoxide anion levels were measured using lucigenin chemiluminescence, as previously described (Martín *et al.* 2005). Briefly, aortic segments were rinsed in KHS for 30 min, equilibrated for 30 min in HEPES buffer at 37 °C, transferred to test tubes that contained 1 ml HEPES buffer (pH 7.4) containing lucigenin (250 µM) and then kept at 37 °C. The luminometer was set to report arbitrary units of emitted light; repeated measurements were collected during 5 min at 10-s intervals and averaged. 4,5-Dihydroxy-1,3-benzene-disulphonic acid 'Tiron' (10 mM), a cell permeant, non-enzymatic scavenger of the superoxide anion, was added to quench the superoxide anion-dependent chemiluminescence. Also blank measures were collected in the same way without aortic segments to subtract background emission. Some assays were performed in the presence of SOD (13 U/ml) to ensure the specificity of the method.

SOD activity

SOD activity was measured by inhibition of pyrogallol autooxidation (Marklund & Marklund 1974). Frozen aortic segments (–70 °C) were homogenated in 50 mM Tris–HCl

buffer (pH 8.2) containing 1 mM metal chelator diethylenetriamine penta-acetic acid (DTPA). After centrifugation at 12 000 *g* for 10 min, 50 μ l supernatants were mixed with 0.45 ml of 50 mM Tris-HCl buffer (pH 8.2) containing 1 mM DTPA and 200 μ M pyrogallol. The change of absorbance at 420 nm was measured after 3 min reaction at room temperature, using a Shimadzu UV-160 A spectrophotometer. The SOD activity was expressed as SOD units per milligram protein, interpolating the results in a known SOD concentration curve.

Vascular reactivity

The method used for isometric tension recording has been previously described completely elsewhere (Nielsen & Owman 1971). Briefly, two parallel stainless steel pins were introduced through the lumen of the vascular segment: one was fixed to the bath wall and the other connected to a force transducer (Grass FTO3C; Grass Instruments Co., Quincy, MA, USA); this was connected in turn to a model 7D Grass polygraph. Segments were suspended in an organ bath containing 5 ml KHS at 37 °C continuously bubbled with a 95% O₂–5% CO₂ mixture (pH 7.4). The segments were subjected to a tension of 1 g, which was readjusted every 15 min during a 90 min equilibration period before drug administration. After this, the vessels were exposed to 75 mM KCl to check their functional integrity. After a washout period, the presence of vascular endothelium was tested by the ability of 10 μ M ACh to relax segments precontracted with 1 μ M noradrenaline (NA).

The segments were rinsed several times with KHS over a 1-h period, and then a cumulative concentration–response curve to ACh (10 nM–10 μ M) or to the NO donor, sodium nitroprusside (SNP, 10 nM–10 μ M) was obtained.

To analyse the possible effects of superoxide anions on the response elicited by SNP, concentration–response curves to the NO donor SNP (10 nM–10 μ M) were performed in endothelially denuded segments from both rat groups precontracted with 1 μ M NA, in the presence of either SOD (13 U/ml) or the membrane-permeable mimetic of SOD, tempol (0.1 mM).

To study the participation of endogenous SOD in the relaxation induced by SNP in both groups of rats, concentration-dependent curves to SNP were performed in the presence of the Cu/Zn-SOD inhibitor diethyldithiocarbamate (DETC; 1 mM) in endothelially denuded segments.

Relaxation was calculated as the percentage inhibition of the initial contraction elicited by 1 μ M NA.

Solutions and drugs

The composition of KHS was as follows (mM): NaCl 115, CaCl₂ 2.5, KCl 4.6, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.2, NaHCO₃ 25, glucose 11.1, Na₂ EDTA 0.03. Drugs used were: L-NA hydrochloride, ACh chloride, SNP, DAF-2, DETC, exogenous SOD and tempol (Sigma–Aldrich). Stock solution (10 mM) of drugs was made in distilled water, except

for NA which was dissolved in NaCl (0.9%)–ascorbic acid (0.01%, w/v) solution. These solutions were kept at –20 °C and appropriate dilutions were made in KHS on the day of the experiment.

Statistical analysis

Results are given as means $\pm$ S.E.M. For the reactivity experiments, statistical analysis was done by comparing the curve obtained in the presence of the different substances with the control curve by means of measure ANOVA (two tails). For the experiments of NO release, superoxide anion production and those of SOD activity, the statistical analysis was done using a Student's *t*-test for unpaired experiments. A *P* value <0.05 was considered significant.

Results

Expression of eNOS

The expression of eNOS was detected in homogenates from fresh rat aortic segments, and it was similar in arteries from control and orchidectomized rats (Fig. 1a). Since orchidectomy did not change aorta thickness (J Blanco-Rivero, C Martin & M Ferrer, unpublished observations), the eNOS expression was normalized to α -actin (Fig. 1b).

NO release

Basal and ACh-induced NO release were similar in aortic segments from control and orchidectomized rats (Fig. 2).

Figure 1 Representative Western blot for (a) eNOS and α -actin expression and (b) densitometric analysis of eNOS expression in aortic segments from control and orchidectomized rats. Results (means $\pm$ S.E.M.) are expressed as the ratio between the signal for eNOS protein and the signal for α -actin. Number of animals is indicated in parenthesis.

Figure 2 Basal and ACh-induced NO release in aortic segments from control and orchidectomized rats. Results (means $\pm$ S.E.M.) are expressed as arbitrary units (A.U.) per milligram tissue. Number of animals is indicated in parenthesis. * $P < 0.05$ compared with basal NO release.

Preincubation with L-NAME (0.1 mM) abolished the NO release in arteries from both groups (data not shown).

Superoxide anion production

Basal superoxide anion production was similar in aortic segments from control or orchidectomized rats, while the ACh-induced anion production was greater in arteries from orchidectomized rats (Fig. 3).

Vascular reactivity

In aortic segments precontracted with NA (0.1 μ M), ACh (0.1 nM–10 μ M) induced a concentration-dependent relaxation that was greater in segments from orchidectomized than in those from control rats (Fig. 4a). However, the relaxation induced by SNP (0.1 nM–10 μ M) was similar in arteries from both groups of rats (Fig. 4b).

Figure 3 Basal and ACh-induced superoxide anion formations in aortic segments from control and orchidectomized rats. Results (means $\pm$ S.E.M.) are expressed as arbitrary units (A.U.) per milligram tissue per minute. Number of animals is indicated in parenthesis. * $P < 0.05$, † $P < 0.01$ compared with basal superoxide anion formation.

To analyse the possible involvement of superoxide anion in the relaxation induced by SNP, the effect of the superoxide anion scavenger, SOD, or the membrane-permeable mimetic of SOD, tempol (0.1 mM), was examined in endothelially denuded aortas. Preincubation with SOD did not modify the SNP-induced relaxation in arteries from control or orchidectomized rats (Fig. 5a and b). Preincubation with tempol increased the SNP-induced relaxation more in aortas from orchidectomized than control rats (Fig. 5a and b). To investigate the participation of endogenous SOD in the relaxation induced by SNP, the effect of Cu/Zn-SOD inhibitor DETC was analysed. Preincubation with 1 mM DETC decreased the relaxation induced by SNP to a similar extent in aortic segments from control and orchidectomized rats (Fig. 5a and b).

Expression of Cu/Zn-SOD

The expression of Cu/Zn-SOD was greater in aortas from orchidectomized than control rats (Fig. 6).

Activity of endogenous SOD

The activity of endogenous SOD was greater in aortas from orchidectomized than control animals (Fig. 7).

Discussion

In a previous study, we described how ACh-induced relaxation was greater in aortas from orchidectomized than control male rats, and showed the involvement of superoxide anion vasodilatory action through BK_{Ca} channel activation (Ferrer *et al.* 1999). Since there is a lack of studies analysing the effect of endogenous male sex hormones on the whole NO pathway in the same experimental conditions, we have now analysed the possible regulation by endogenous male sex hormones of: (1) eNOS expression, (2) eNO release induced by ACh and (3) NO metabolism, taking into account both the production and the removal of superoxide anion.

In regards to the influence of androgens on eNOS expression, increase (Simoncini *et al.* 2003), decrease (Chatrath *et al.* 2003) or no change (McNeill *et al.* 1999) have all been reported. We found that aortic eNOS expression was not modified by orchidectomy as has been observed in rat mesenteric artery (Blanco-Rivero *et al.* 2006), but in contrast to observations of nNOS expression in rat mesenteric artery (Martín *et al.* 2005), thus indicating that endogenous male sex hormones act differently, depending on tissue and target protein.

Therefore, the next step was to analyse the NO release synthesized by eNOS in response to ACh in aortas from control and orchidectomized rats. We observed that the formation of eNO was not modified by orchidectomy, as also observed with eNOS in mesenteric arteries (Blanco-Rivero *et al.* 2005), although increase (Liu & Dillon 2002, Simoncini

Figure 4 Vasorelaxant response to (a) ACh and (b) SNP in aortic segments from control and orchidectomized rats. Results (means $\pm$ S.E.M.) are expressed as percentage of inhibition of contraction induced by $1 \mu\text{M}$ NA. Number of animals is indicated in parenthesis.

et al. 2003) and decrease (Mukherjee *et al.* 2001) of eNOS activity by androgens have been reported. These differences could be due to differences in the tissues analysed, time of androgen treatment and/or type of stimulus applied to induce NO release.

It is known that reactive oxygen species are involved in metabolising NO (Gryglewski *et al.* 1986, Ferrer *et al.* 2000, 2001). Among all the reactive oxygen species, superoxide anion plays a critical role since it is a source of many other reactive oxygen intermediates (Beckman & Koppenol 1996). In a previous work, we found a dual behaviour of superoxide anion depending on the presence/absence of endogenous male sex hormones, in such a way that in aortas from control rats superoxide anions were involved in metabolising the ACh-induced NO, while in arteries from orchidectomized rats superoxide anion-induced vasorelaxation through K_{Ca} channels activation (Ferrer *et al.* 1999). These findings would

explain why the sensitivity of smooth muscle cells to eNO was similar in aortas from control and orchidectomized rats; despite the ACh-induced vasodilator response was greater in aortas from orchidectomized rats (Ferrer *et al.* 1999). We now analysed the superoxide anion formation in aortas from control and orchidectomized rats. The lucigenin chemiluminescence measurement showed that ACh-induced formation of superoxide anion was greater in segments from orchidectomized than control rats. This result agrees with a study carried out in mesenteric artery (Martín *et al.* 2005) and with previous studies showing the antioxidant properties of androgens (Békési *et al.* 2000, Yorek *et al.* 2002).

It has been widely reported that superoxide anion, as well as other reactive oxygen species, can induce oxidative processes associated with cardiovascular disorders (Harrison 1994, Munzel *et al.* 1997). Therefore, both production and removal of superoxide anion are important contributors to the

Figure 5 Effect of SOD, tempol and DETC on the concentration-response curves to SNP in endothelium denuded aortic segments from (a) control and (b) orchidectomized rats. Results (means $\pm$ S.E.M.) are expressed as percentage of inhibition of contraction induced by $1 \mu\text{M}$ NA. Number of animals is indicated in parenthesis.

Figure 6 Representative Western blot for (a) Cu/Zn-SOD and α -actin expression and (b) densitometric analysis of Cu/Zn-SOD expression in aortic segments from control and orchidectomized rats. Results (means $\pm$ S.E.M.) are expressed as the ratio between the signal for Cu/Zn-SOD protein and the signal for α -actin. Number of animals is indicated in parenthesis. * $P < 0.01$ compared with control rats.

maintenance of appropriate levels of this oxygen species. We have described above the differences in the superoxide production induced by ACh, associated with endogenous male sex hormones presence or absence. However, no data are available about the role of endogenous male sex hormones on mechanisms involved in the elimination of superoxide anion. Within the vessel wall, SODs transform superoxide anion to hydrogen peroxide (Oury *et al.* 1996, Price *et al.* 2000, Muzykantov 2001). Although three SOD isoforms have been identified: cytosolic Cu/Zn-SOD, mitochondrial

Figure 7 SOD activity in aortic segments from control and orchidectomized rats. Results (means $\pm$ S.E.M.) are expressed as SOD units per milligram protein. Number of animals is indicated in parenthesis. * $P < 0.01$ compared with control rats.

MnSOD and extracellular ecSOD – which is also Cu/Zn-dependent (Strehlow *et al.* 2003), we have focused on analysing the participation of Cu/Zn-SOD, since it is the predominant isoform in peripheral vessels (Namgaladze *et al.* 2005), and this enzyme therefore plays a crucial role in the pathogenesis of vascular dysfunction (Wolin 2002).

The next step was to compare the possible functional role of superoxide anion and endogenous SOD in vessels from orchidectomized and control rats. Since the modulation of eNO release by K channels activation has been reported (Qiu & Quilley 2001, Gendron *et al.* 2004), and taking into account that we have previously demonstrated the BK_{Ca} channel activation by superoxide anion (Ferrer *et al.* 1999), to avoid misinterpretation the vascular function of these anions was analysed in endothelium-denuded aorta.

The fact that DETC strongly inhibited SNP-induced relaxation, suggest an active role for endogenous SOD. To investigate the possible differences in the functional involvement of superoxide anion in the two groups of rats, we analysed the effect of the superoxide anion scavengers, exogenous SOD and the membrane-permeable mimetic of SOD, tempol, on the relaxation induced by SNP. We observed that exogenous SOD did not induce modification in the SNP-induced vasorelaxation in either control or orchidectomized rats. However, tempol induced slight or strong increase in the vasodilator response to SNP respectively in arteries from control or orchidectomized rats. These results indicate a higher NO metabolism in arteries from orchidectomized rats, as we have found in mesenteric artery (Blanco-Rivero *et al.* 2005) and reinforced the described antioxidant properties of androgens (Békési *et al.* 2000, Yorek *et al.* 2002, Martín *et al.* 2005). Additionally, the lack of effect of exogenous SOD would indicate that the predominant isoform involved in this effect would be the cytosolic Cu/Zn-SOD.

On the other hand, there are reports describing an increased expression and/or activity of SOD in cardiovascular pathologies, in which overproduction of superoxide anion exists (Kobayashi *et al.* 2002, Tanaka *et al.* 2005). Despite the increased superoxide anion production in arteries from orchidectomized rats, which is a risk factor for the development of cardiovascular dysfunction, to our knowledge, there is no information about modulation of SOD expression or activity by endogenous male sex hormones in peripheral tissues, in contrast to oestrogen studies, which report increased SOD activity (Strehlow *et al.* 2003). Therefore, the next step was to analyse the possible modification by endogenous male sex hormones of Cu/Zn-SOD expression and activity. We found that both the expression and activity of Cu/Zn-SOD were increased in aortic segments from orchidectomized rats. These results indicate that the increases in both expression and activity of Cu/Zn-SOD could be a compensatory mechanism to eliminate the elevated superoxide anion formation induced by orchidectomy in male rats.

The findings of this study seem to be physiologically relevant, since although Cu/Zn-SOD expression and activity

are increased in aortas from orchidectomized rats, superoxide anion levels are elevated enough to metabolize eNO. This situation could indicate a disadvantage in cardiovascular function in the absence of male sex hormones, thereby suggesting cardioprotective effects of androgens.

In conclusion, the results obtained in the present work show that endogenous male sex hormone deprivation altered neither eNOS expression nor eNO release; however, it increased the expression and activity of Cu/Zn-SOD in an attempt to compensate for increased superoxide anion formation. However, the predominant vascular effect of orchidectomy is to decrease the NO bioavailability.

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