Protein kinase C activation increases endothelial nitric oxide release in mesenteric arteries from orchidectomized rats

Javier Blanco-Rivero, Ana Sagredo, Gloria Balfagón and Mercedes Ferrer

Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma de Madrid, C/Arzobispo Morcillo 4, 28029 Madrid, Spain

(Requests for offprints should be addressed to M Ferrer; Email: mercedes.ferrer@uam.es)

Abstract

The aim of the present study was to assess the effect of endogenous male sex hormones on endothelial nitric oxide synthase (eNOS) expression, release and function of the endothelial nitric oxide (NO), as well as to assess the regulatory action of protein kinase C (PKC) on acetylcholine (ACh)-induced endothelial NO release. For this purpose, superior mesenteric arteries from control and orchidectomized male Sprague–Dawley rats were used. eNOS expression and basal- and ACh-induced NO release were similar in arteries from both groups of rats. Orchidectomy decreased the vasodilator effect induced by ACh but did not alter that induced by sodium nitroprusside (SNP). The superoxide anion scavenger, superoxide dismutase (SOD), or the membrane-permeable mimetic of SOD, tempol, only enhanced ACh-induced relaxation in arteries from orchidectomized rats. ACh-induced TXA₂ formation was higher in arteries from orchidectomized than from control rats. Neither the PKC activator, phorbol 12,13-dibutyrate (PDBu), nor the non-selective PKC inhibitor, calphostin C, modified basal- or ACh-induced NO release in arteries from control rats. In arteries from orchidectomized rats, basal- and ACh-induced endothelial NO release were

increased by PDBu but decreased by calphostin C. Both Gö6976, a PKC inhibitor that is partially selective for conventional PKC isoforms, as well as PKCζ pseudosubstrate inhibitor (PKCζ-PI) decreased both basal- and ACh-induced NO release in arteries from orchidectomized rats. Neither PDBu nor calphostin C modified the vasodilator response induced by ACh in arteries from control rats. In segments from orchidectomized rats, PDBu enhanced the ACh-induced response, but this response was not modified by calphostin C, Gö6976 or PKCζ-PI. The vasodilator response induced by SNP was not altered by the PKC activators or inhibitors in any artery from either group. These results show that endogenous male sex hormone deprivation does not affect the eNOS expression or the endothelial NO release induced by ACh, but does decrease the vasodilator action of ACh, by increasing NO metabolism and TXA₂ formation. In addition, PKC seems to modulate eNOS activity only in mesenteric arteries from orchidectomized rats, in which conventional and PKCζ isoforms are involved in the positive regulation of eNOS.

Journal of Endocrinology (2007) **192**, 189–197

Introduction

The role of androgens in vascular function is receiving considerable research interest (Sader & Celermajer 2002) since recent studies have reported some protective actions of androgens in males (Liu *et al.* 2003, Perusquia 2003, Littleton-Kearney & Hurn 2004). One of the interesting factors is the proposed interaction between androgens and endothelial nitric oxide (NO; Tep-areenan *et al.* 2003, Jones *et al.* 2004). In this regard, most studies have been focused on analysing the effects of androgenic derivatives on different aspects of the NO system, such as endothelial nitric oxide synthase (eNOS) expression, NO release or NO vasodilator effect (Hutchison *et al.* 1997, Ceballos *et al.* 1999, Teoh *et al.* 2000). However, there is a lack of experimental data about the specific effect of endogenous male sex hormone on these aspects when they are studied simultaneously, as has been done for neuronal nitric oxide (Martín *et al.* 2005).

The functional role of endothelial NO in vascular tone regulation has been widely reported (Furchgott & Zawadzki 1980, Sánchez-Ferrer & Marín 1990, Vanhoutte 1996). Endothelial NO is formed by eNOS (Förstermann *et al.* 1991, Pollock *et al.* 1991, 1993), which is regulated by multiple kinases (Dinerman *et al.* 1994, Michell *et al.* 2001). Several studies have reported that protein kinase C (PKC) could phosphorylate eNOS and modulate its activity, thereby leading to a decrease (Mukherjee *et al.* 2001, Yakubu *et al.* 2004) or increase (Li *et al.* 1998, Wedgwood *et al.* 2001) of endothelial NO production. In this sense, we observed that male sex hormone deprivation increased PKC activity of rat mesenteric arteries, and this positively modulates neuronal NO release in these arteries (Blanco-Rivero *et al.* 2005a,b).

With this in mind, the aim of the present study is to determine whether endogenous male sex hormones influence eNOS expression and activity, and thereby the functionality of

the released endothelial NO. The possible regulatory action of PKC on endothelial NO release and on its vasodilator effect will be also analysed.

Materials 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. Male sex hormone deprivation was induced by gonadectomy at 7 weeks of age, and 4 months later the animals were killed. The observation of seminal vesicles atrophy confirmed successful surgery. Rats were killed by CO₂ inhalation; the first branch of the mesenteric artery was carefully dissected out, cleaned of connective tissue and placed in Krebs–Henseleit solution (KHS, in 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) 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). This study was also approved by the Ethical Committee of Universidad Autónoma de Madrid.

Western blot analysis of endothelial NOS expression

For western blot analysis of eNOS protein expression, superior mesenteric arteries were homogenized in a boiling buffer composed of 1 mM sodium vanadate (a protease inhibitor), 1% SDS and 0.01 M Tris–HCl (pH 7.4). Homogenates containing 16.5 µg protein were electrophoretically separated on a 7.5% SDS–PAGE and then transferred to polyvinylidene difluoride membranes (Bio–Rad Immun–Blot overnight at 4 °C, 230 mA), using a Bio–Rad Mini Protean III system (Bio–Rad Laboratories) containing 25 mM Tris, 190 mM 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 3 h at room temperature in Tris–buffered saline solution (100 mM, 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). After washing, the membrane was incubated with a 1:2000 dilution of antimouse immunoglobulin G antibody 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, National Institute of Health, Bethesda, MD, USA). The same membrane, after stripping (3 h at 60 °C in 100 mM β-mercaptoethanol, 2% SDS and 0.06 M Tris–HCl (pH 6.8)) and extensively washing, was used to determine α-actin expression, and the content of the latter was used to correct eNOS expression in each sample by means of a monoclonal antibody anti α-actin (1:2000 dilution, Sigma).

Nitric oxide release

Mesenteric arteries from control and orchidectomized rats were subjected to a resting tension of 0.5 g, as indicated for the reactivity experiments. 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 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. The validation of this method has been studied by comparing the results obtained with DAF with those obtained by nitrites measurement (Martín *et al.* 2005).

The interference of phorbol dibutyrate (PDBu, a PKC activator) or calphostin C (a non-selective PKC inhibitor), Gö6976 (a PKC inhibitor that is partially selective for conventional over novel PKC isoforms) or PKCζ pseudosubstrate inhibitor (PKCζ-PI) on NO release was studied by incubating the arteries with these drugs 30 min before collecting the medium to determine basal NO release, or 30 min prior to the ACh addition for determination of ACh-induced NO release. The ACh-induced NO release was calculated by subtracting basal NO release from that evoked by ACh. Also, blank measures were collected in the same way from segment-free medium in order to subtract background emission. Some assays were performed in the presence of N^G-nitro-L-arginine (L-NAME; 0.1 mM) to ensure the specificity of the method. The amount of NO released was expressed as arbitrary units/mg tissue.

Vascular reactivity

The method used to record isometric tension recording has been described in full 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 0.5 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 confirmed 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, and then cumulative concentration–response curves to ACh (0.1 nM–10 μ M) or the NO donor sodium nitroprusside (SNP, 10 nM–10 μ M) were obtained.

To analyse the participation of superoxide anion on the response elicited by ACh, curves with this agent were performed with segments from both groups of rats in the presence of the superoxide anion scavenger, superoxide dismutase (SOD, 13 U/ml), or in the presence of the membrane-permeable mimetic of SOD, tempol, (0.1 mM).

To analyse the involvement of PKC in ACh-induced response, either 1 nM PDBu or 0.1 μ M calphostin C was added to the bath 30 min before concentration–response curves to ACh (0.1 nM–10 μ M) were performed in arteries from both the control and the orchidectomized rats. To analyse the PKC isoform involved in the ACh-induced response in arteries from orchidectomized rats, 1 μ M Gö6976 or 10 μ M PKC ζ -PI were added to the bath 30 min before concentration–response curves to ACh (0.1 nM–10 μ M) were performed. The effect of PDBu plus 0.1 mM L-NAME on the concentration–response curve to ACh was performed in arteries from orchidectomized rats.

To analyse the possible effects of PDBu or calphostin C on the response induced by NO, concentration–response curves to SNP (0.1 nM–10 μ M) were performed in segments from both rat groups precontracted with 1 μ M NA, in the presence or absence of PDBu or calphostin C. Additionally, the effect of 1 μ M Gö6976 or 10 μ M PKC ζ -PI on the concentration–response curve to SNP was tested in segments from orchidectomized rats.

TXA₂ production

The production of thromboxane A (TXA₂) *in vivo* is typically monitored by measuring the stable metabolite thromboxane B (TXB₂), using a TXB₂ immunoassay (R&D Systems). Segments of rat mesenteric artery were preincubated for 30 min in 5 ml KHS at 37 °C, continuously gassed with 95% O₂–5% CO₂ mixture. After several 10-min washout periods in a bath of 0.2 ml KHS, the medium was collected to measure basal TXB₂ release. Once the chamber was refilled, the ACh concentration curve (0.1 nM–10 μ M) was applied at 2-min intervals. The assay was made following the manufacturer's instructions. Briefly, samples were transferred to a 96-well plate coated with goat anti-rabbit polyclonal antibody and incubated at room temperature for 2 h with a specific rabbit polyclonal antibody to TXB₂. After washing, a solution of *p*-nitrophenyl phosphate was added for 45 min at room temperature, to reveal the reaction. Then, the reaction was stopped and the plate was read at 405 nm using a microplate reader. A blank well was run to

subtract the background signal. Results were expressed as pg TXB₂/mg tissue.

Solutions and drugs

The following drugs were used in the experiment: L-NA hydrochloride; ACh chloride; L-NAME hydrochloride; sodium nitroprusside; DAF-2; PDBu acetate; calphostin C; Gö6976; PKC ζ pseudosubstrate inhibitor; SOD and tempol (Sigma-Aldrich). Stock solutions (10 mM) of drugs were made in distilled water, with the exception of NA, which was dissolved in NaCl (0.9%)–ascorbic acid (0.01% w/v) solution, and PDBu, which was dissolved in dimethylsulphoxide. It was observed that the dimethylsulphoxide vehicle did not alter basal tone or the ACh-induced responses. All these solutions were stored at –20 °C and appropriate dilutions were made in KHS on the day of the experiment.

Statistical analysis

The responses elicited by KCl were expressed in mg for comparison between control and orchidectomized rats. The relaxation induced by ACh and SNP were expressed as a percentage of the initial contraction elicited by NA. Results are given as mean $\pm$ S.E.M. In order to compare the magnitude of the effect of NOS blockade on the ACh-induced response, some results were expressed as 'differences' of area under the concentration–response curve (dAUC) in the absence and presence of L-NAME. AUCs were calculated from the individual concentration–response curve plot and the differences were expressed as a percentage of the difference to UCA for segments under the control situation. Statistical analysis was done by comparing the curve obtained in the presence of different substances with the control curve by means of ANOVA (two tails). For the NO release experiments, the statistical analysis was done using the Student's *t*-test for unpaired experiments. A *P* value of less than 0.05 was considered significant.

Results

Expression of endothelial NOS

The expression of eNOS, normalized to α -actin, was similar in mesenteric artery homogenates from control or orchidectomized rats (Fig. 1).

NO release

Basal- and ACh-induced NO release were similar in mesenteric arteries from both control and orchidectomized rats (ANOVA, *P* > 0.05). Preincubation with L-NAME (10 μ M) abolished the NO release in arteries from both groups (data not shown). Preincubation with PDBu (1 nM) or calphostin C (0.1 μ M) failed to modify the basal- and the

Figure 1 Representative western blots for eNOS and α -actin expression (a) and densitometric analysis of eNOS expression (b) in mesenteric artery segments from control and orchidectomized male rats. Results (mean $\pm$ 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 parentheses.

ACh-induced NO release in arteries from control male rats (Fig. 2). In arteries from orchidectomized rats, preincubation with PDBu (1 nM) increased the basal- and the ACh-induced NO release, while calphostin C (0.1 μ M), Gö6976 (1 μ M) or PKC ζ -PI (10 μ M) decreased them (Fig. 2).

Vascular reactivity

The vasodilator response induced by ACh was greater in mesenteric arteries from control than from orchidectomized rats (ANOVA, $P < 0.01$; Fig. 3). The vasodilator response

induced by SNP was similar in mesenteric arteries from both groups (ANOVA, $P > 0.05$; Fig. 3).

The ACh-induced response was equally decreased by preincubation with the NOS inhibitor L-NAME (10 μ M) in segments from both control or orchidectomized rats, as also indicated by the dAUC to ACh in the absence and presence of L-NAME in arteries from control and orchidectomized rats (Fig. 4).

Preincubation with SOD (13 U/ml) or tempol (0.1 mM) did not modify the relaxation induced by ACh in segments from control rats, but it increased the relaxation induced by ACh in arteries from orchidectomized rats (Fig. 5).

Neither the presence of calphostin C (0.1 μ M) nor PDBu (1 nM) modified the vasodilator response induced by ACh or SNP in arteries from control rats (data not shown). However, in segments from orchidectomized animals, the response induced by ACh was enhanced by PDBu but unaffected by calphostin C, Gö6976 or PKC ζ -PI (Fig. 6). The concentration-response curve to SNP (0.1 nM–10 μ M) was not altered by the PKC activators or PKC inhibitors used in arteries from orchidectomized rats (Fig. 6).

In segments from orchidectomized rats, preincubation with L-NAME strongly decreased the ACh-induced relaxation, and the presence of PDBu did not modify that effect (data not shown).

The drugs used did not alter the basal tone in segments from either group. The NA precontraction was not modified by calphostin C (0.1 μ M) or PDBu (1 nM) in either group of rats (data not shown).

TXA₂ production

Basal TXB₂, the stable TXA₂ metabolite, was increased in mesenteric arteries from orchidectomized rats compared with control rats (Table 1). Stimulation with ACh (0.1 nM–10 μ M)

Figure 2 Effect of PDBu (1 nM) or calphostin C (Calph C; 0.1 μ M) on the basal- and ACh-induced NO release in mesenteric artery segments from control and orchidectomized animals. Results (mean $\pm$ S.E.M.) are expressed as arbitrary units/mg tissue. n , number of animals. * $P < 0.05$, † $P < 0.01$ versus the respective basal NO release; * $P < 0.05$ versus basal NO release in non-treated arteries.

Figure 3 Effect of orchidectomy on the concentration–response curve to acetylcholine (ACh) and sodium nitroprusside (SNP) in mesenteric artery segments from control and orchidectomized male rats. Results (mean $\pm$ S.E.M.) are expressed as a percentage of a previous tone with noradrenaline. Number of animals is indicated in parentheses.

increased TXB₂ production more in arteries from orchidectomized than from control rats (Table 1).

Discussion

Numerous studies about the effects of androgens on vascular function have been published (Liu *et al.* 2003, Perusquia 2003, Tep-areenan *et al.* 2003, Littleton-Kearney & Hurn 2004), and there is particular interest concerning the endothelial NO system, in light of the relevant functional role of the endothelial NO on regulating vascular tone (Sánchez-Ferrer & Marín 1990, Vanhoutte 1996). Most of the studies have focused on

analysing a single isolated aspect of that system. Thus, it has been reported that androgenic derivatives can increase (Simoncini *et al.* 2003), decrease (Chatrath *et al.* 2003) or not affect (McNeill *et al.* 1999) eNOS expression. Regarding eNOS activity, androgen-induced increases (Liu & Dillon 2002, Simoncini *et al.* 2003) and decreases (Mukherjee *et al.* 2001) have both been reported. Other researchers have demonstrated that androgens increased (Wynne & Khalil 2003, Orshal & Khalil 2004) and decreased (Ba *et al.* 2001, Gonzales *et al.* 2004) the vasodilator effect of endothelial NO. Despite all these findings, there is a lack of systematic studies analysing the effect of endogenous male sex hormones on all these aspects taken together, in a manner similar to that

Figure 4 Effect of the NOS inhibitor, L-NAME, on the concentration–response curve to acetylcholine (ACh) in mesenteric artery segments from control and orchidectomized rats. Results (mean $\pm$ S.E.M.) are expressed as a percentage of a previous tone with noradrenaline. Number of animals is indicated in parentheses. Insert graph shows dAUC to ACh in the absence and presence of L-NAME from control (C) and orchidectomized (CX) rats. dAUC values (mean $\pm$ S.E.M.) are expressed as a percentage of the difference to UAC for segments under the control situation.

Figure 5 Effect of SOD or tempol on the concentration–response curve to acetylcholine (ACh) in mesenteric artery segments from control and orchidectomized rats. Results (mean $\pm$ s.e.m.) are expressed as a percentage of a previous tone with noradrenaline. Number of animals is indicated in parentheses.

performed with neuronal nitric oxide synthase (nNOS) expression, neuronal NO release and vasodilator effect (Martín *et al.* 2005).

We found that eNOS expression was not modified by orchidectomy in rat mesenteric arteries in contrast to our observation of nNOS in the same artery (Martín *et al.* 2005), which indicates that endogenous male sex hormones act in a different manner depending on the target protein. Therefore, the next step was to analyse the NO release formed by ACh-induced eNOS stimulation in arteries from control and orchidectomized rats. We found that orchidectomy did not modify endothelial NO formation. However, although the same amount of NO was released in response to ACh, the vasodilator response elicited by this agent was greater in arteries from control than from orchidectomized rats. The

vasodilator action of ACh is known to depend on several endothelial factors, whose release is time and concentration dependent (Sunano *et al.* 2001, Sekiguchi *et al.* 2002, Chauhan *et al.* 2003). In view of this puzzling response, we tried to ascertain the degree of participation by endothelial NO in the vasodilator effect of ACh by analysing the ACh-induced response in the presence of the NOS inhibitor, L-NAME. We observed that L-NAME decreased the vasodilator response induced by ACh to a similar extent in arteries from both groups of animals, indicating a similar contribution of endothelial NO in arteries from control and orchidectomized rats. Therefore, the differences in the ACh-induced vasodilation between arteries from control and orchidectomized rats could be due to differences in (1) the sensitivity of smooth muscle cells to endothelial NO, (2) the

Figure 6 Effect of PDBu, calphostin C (Calph C), Gö6976 or PKC ζ -PI on the concentration–response curves to acetylcholine (ACh) and sodium nitroprusside (SNP) in mesenteric artery segments from orchidectomized rats. Results (mean $\pm$ s.e.m.) are expressed as a percentage of a previous tone with noradrenaline. Number of animals is indicated in parentheses.

Table 1 Basal- and acetylcholine-induced TXB₂ release in rat mesenteric artery segments from control and orchidectomized rats. Results (mean ± s.e.m.) are expressed as pg TXB₂/ml mg tissue. Number of animals is in parentheses

	Basal	ACh-induced
Control (4)	299.3 ± 100.1	2007.0 ± 342.0*
Orchidectomized (4)	886.3 ± 213.4 [†]	3118.0 ± 337.7* [†]

* $P < 0.05$ versus basal TXB₂ release, [†] $P < 0.01$ versus TXB₂ release in control rats.

NO metabolism and/or (3) an imbalance between endothelial vasodilator/vasoconstrictor substances that would counteract the vasodilator effect of ACh. The fact that the vasodilator response to the NO donor SNP was similar in arteries from orchidectomized and control rats ruled out different sensitivities to the NO released in response to ACh. In addition, we observed that superoxide anion negatively modulates the vasodilator response to ACh only in arteries from orchidectomized rats, since SOD, a superoxide anion scavenger, or tempol, a membrane-permeable mimetic of endogenous SOD, increased the relaxation to ACh only in arteries from these rats. These results indicate a greater NO metabolism in arteries from orchidectomized rats and are in line with reports describing the antioxidant properties of androgens (Békési *et al.* 2000, Yorek *et al.* 2002, Martín *et al.* 2005). Additionally, these results also indicate that the localization of the SOD involved in this effect would be predominantly extracellular.

Despite these findings, the involvement of other vasoactive substances that counteract the vasodilator effect of ACh cannot be ruled out. Since we have also observed that the basal release of TXA₂ is increased in mesenteric arteries from orchidectomized rats (Blanco-Rivero *et al.* 2005a,b), we analysed the TXA₂ production induced by ACh. We found that ACh-induced TXA₂ formation was greater in arteries from orchidectomized than from control rats, indicating the involvement of this vasoconstrictor prostanoid in the response to ACh.

The results observed at this point showed that orchidectomy does not affect either eNOS expression or basal- or ACh-induced NO release, but it does decrease the vasodilator effect induced by ACh, by increasing NO metabolism and TXA₂ production.

Since we have reported that orchidectomy increased PKC activity in rat mesenteric arteries and that PKC positively regulates nNOS activity (Blanco-Rivero *et al.* 2005a,b), our second objective was to analyse the influence of endogenous male sex hormones on the PKC regulation of eNOS activity.

PKC is a family of phospholipid-dependent serine-threonine kinases that are critical for signal transduction of a wide range of biological responses (Nishizuka 1984, Shen 2003) through actions on ion channels, transcription factors, other kinases and still other proteins (Spitales & Cantrell 2004, Ward *et al.* 2004). In this sense, it has been reported that PKC, depending on the tissue analysed, positively (Li *et al.* 1998, Wedgwood *et al.* 2001) or negatively (Mukherjee *et al.* 2001,

Yakubu *et al.* 2004) regulates eNOS activity. PKC inhibition has also been reported to increase eNOS activity (Li *et al.* 2005). Therefore, the next step was to analyse how PKC activation or inhibition regulates eNOS activity in arteries from orchidectomized and control animals. We found that neither the PKC activator, PDBu (Nishizuka 1984, Ferrer *et al.* 1992), nor the PKC inhibitor, calphostin C (Kobayashi *et al.* 1989, Ferrer *et al.* 2000), modified ACh-induced NO release in arteries from control animals. In contrast, in arteries from orchidectomized rats, PKC activation increased both basal- and ACh-induced NO release, while PKC inhibition strongly decreased both basal- and ACh-induced NO release. These results indicate that PKC participates in eNOS activity only in arteries from orchidectomized rats, and also seem to indicate that PKC apparently did not regulate eNOS activity in arteries from control animals, which contrasts with our results on nNOS (Blanco-Rivero *et al.* 2005a,b) in which we observed nNOS activity modulation by PKC. In this respect, it is possible to speculate that very subtle differences could exist in the modulation of NOS isoforms, i.e. regulatory mechanisms other than PKC could be working on eNOS, including different redox conditions (Polytarchou & Papadimitriou 2005), phosphatase activity (Fleming *et al.* 2001) and/or other kinases (Ferrer *et al.* 2004) that, in turn, regulate the intracellular environment and function.

Additionally, since the pharmacological mimic of diacylglycerol, PDBu, stimulates conventional and novel PKC isoforms (Ward *et al.* 2004) and since calphostin C is a non-specific PKC inhibitor, we tested the effect of the PKC inhibitor Gö6976, which is partially selective for conventional over novel and atypical PKC isoforms (Martiny-Baron *et al.* 1993, Ward *et al.* 2004). We observed that this inhibitor also decreased basal- and ACh-induced NO in arteries from orchidectomized rats, indicating the involvement of the conventional PKC isoforms in endothelial NO regulation. Since the atypical PKC ζ isoform has been reported to modulate vascular responses (Damron *et al.* 1998, De Witt *et al.* 2001, Cogolludo *et al.* 2003) and neuronal NO release (Blanco-Rivero *et al.* 2005a,b), we also tested the possible involvement of this isoform in endothelial NO release. The fact that PKC ζ -PI decreased the basal- and ACh-induced NO release showed the participation of this isoform. Moreover, since the three PKC inhibitors that we used, calphostin C, Gö6976 and PKC ζ -PI, decreased both basal- and ACh-induced NO release, it seems that eNOS, like nNOS (Blanco-Rivero *et al.* 2005a,b), would have already been activated by PKC in arteries from orchidectomized rats.

To investigate the possible functional role of PKC in the endothelially released NO in arteries from both orchidectomized and control rats, we analysed the effect of PKC activation and inhibition on the vasomotor responses induced by ACh. In segments from control rats, preincubation with either the PKC activator or the inhibitor, PDBu or calphostin C respectively did not modify the vasodilator response to ACh, which is in line with the results on NO release. The fact that the response to SNP was not altered by PDBu or

calphostin C also indicates that the vasodilator effect of the released NO is not affected.

However, in segments from orchidectomized rats, PDBu increased the relaxation induced by ACh but did not modify SNP-induced relaxation. These results are in line with the data showing that PDBu increased NO release without altering the NO response. In addition, the fact that PDBu, in the presence of L-NAME, was not able to further increase the relaxation induced by ACh rules out the participation of other vasodilator substances whose synthesis could be increased by PKC activation (Schmeck *et al.* 2003, Eligini *et al.* 2005). Since the PKC inhibitors calphostin C, Gö6976 and PKC ζ -PI decreased the NO release induced by ACh but did not modify the ACh- or SNP-induced vasodilator response, these indicate that the NO released in the presence of the PKC inhibitors would be elevated enough to maintain the vasodilator response to ACh.

In summary, the results obtained in the present work demonstrate that endogenous male sex hormone deprivation does not affect either the eNOS expression or the endothelial NO release induced by ACh, but it does decrease the vasodilator action of ACh by increasing NO metabolism and TXA₂ formation. In addition, PKC only modulates eNOS activity in mesenteric arteries from orchidectomized rats, in which conventional and PKC ζ isoforms are involved in the positive regulation of eNOS.

Acknowledgements

This research was supported by the Fondo de Investigaciones Sanitarias (FIS, PI051767 and PI020335) and the Dirección General de Ciencia y Tecnología (DGCYT, BFI2001-1324). We thank the veterinarian Dr M^a del Carmen Fernández Criado for the care of animals and Ms Ana Pastor and Ms Verónica Sánchez for their technical assistance. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Ba ZF, Wang P, Koo DJ, Ornan DA, Bland KI & Chaudry IH 2001 Attenuation of vascular endothelial dysfunction by testosterone receptor blockade after trauma and hemorrhagic shock. *Archives of Surgery* **136** 1158–1163.
- Békési G, Kakucs R, Várviró S, Racz K, Sprintz D, Feher J & Szekacs B 2000 *In vitro* effects of different steroid hormones on superoxide anion production of human neutrophil granulocytes. *Steroids* **65** 889–894.
- Blanco-Rivero J, Balfagón G & Ferrer M 2005a Male castration increases neuronal nitric oxide synthase activity in the rat mesenteric artery through protein kinase C activation. *Journal of Vascular Research* **42** 526–534.
- Blanco-Rivero J, Balfagón G & Ferrer M 2005b Orchidectomy modulates α_2 -adrenoceptor reactivity in rat mesenteric artery through increased thromboxane A₂ formation. *Journal of Vascular Research* **43** 101–108.
- Ceballos G, Figueroa L, Rubio I, Gallo G, García A, Martínez A, Yanez R, Perez J, Morato T & Chamorro G 1999 Acute and nongenomic effect of testosterone on isolate and perfused rat heart. *Journal of Cardiovascular Pharmacology* **33** 691–697.
- Chatrath R, Ronningen KL, Severson SR, LaBrecche P, Jayachandran M, Bracamonte MP & Miller VM 2003 Endothelium-dependent responses in coronary arteries are changed with puberty in male pigs. *American Journal of Physiology* **285** H1168–H1176.
- Chauhan S, Rahman A, Nilsson H, Clapp L, MacAllister R & Ahluwalia A 2003 NO contributes to EDHF-like responses in rat small arteries: a role for NO stores. *Cardiovascular Research* **57** 207–216.
- Cogolludo A, Moreno L, Bosca L, Tamargo J & Perez-Vizcaino F 2003 Thromboxane A₂-induced inhibition of voltage-gated K⁺ channels and pulmonary vasoconstriction: role of protein kinase C ζ . *Circulation Research* **93** 656–663.
- Damron DS, Nadim HS, Hong SJ, Darvish A & Murray PA 1998 Intracellular translocation of PKC isoforms in canine pulmonary artery smooth muscle cells by ANG II. *American Journal of Physiology* **274** L278–L288.
- De Witt BJ, Kaye AD, Ibrahim IN, Bivalacqua TJ, D'Souza FM, Banister RE, Arif AS & Nossaman BD 2001 Effects of PKC isozyme inhibitors on constrictor responses in the feline pulmonary vascular bed. *American Journal of Physiology* **280** L50–L57.
- Dinerman JL, Steiner JP, Dawson TM, Dawson V & Snyder SH 1994 Cyclic nucleotide dependent phosphorylation of neuronal nitric oxide synthase inhibits catalytic activity. *Neuropharmacology* **33** 1245–1251.
- Eligini S, Barbieri SS, Cavalca V, Camera M, Brambilla M, Fanceschi MD, Tremoli E & Colli S 2005 Diversity and similarity in signalling events leading to rapid Cox-2 induction by tumor necrosis factor- α and phorbol ester in human endothelial cells. *Cardiovascular Research* **65** 683–693.
- Ferrer M, Encabo A, Marín J, Peiro C, Redondo J, de Sagarra MR & Balfagón G 1992 Comparison of the vasoconstrictor responses induced by endothelin and phorbol 12,13-dibutyrate in bovine cerebral arteries. *Brain Research* **599** 186–196.
- Ferrer M, Marín J & Balfagón G 2000 Diabetes alters neuronal nitric oxide release from rat mesenteric arteries. Role of protein kinase C. *Life Sciences* **66** 337–345.
- Ferrer M, Sánchez M, Martín MC, Márquez-Rodas I, Alonso MJ, Salasces M & Balfagón G 2004 Protein kinase A increases electrical stimulation-induced neuronal nitric oxide release in rat mesenteric artery. *European Journal of Pharmacology* **487** 167–173.
- Fleming I, Fisslthaler B, Dimmeler S, Kemp BE & Busse R 2001 Phosphorylation of Thr(495) regulates Ca(2+)/calmodulin-dependent endothelial nitric oxide synthase activity. *Circulation Research* **88** E68–E75.
- Forstermann U, Pollock JS, Schmitt HHHW, Heller M & Murad F 1991 Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelium cells. *PNAS* **88** 1788–1792.
- Furchgott RF & Zawadzki JV 1980 The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288** 373–376.
- Gonzales RJ, Krause DN & Duckles SP 2004 Testosterone suppresses endothelium-dependent dilation of rat middle cerebral arteries. *American Journal of Physiology* **286** H552–H560.
- Hutchison SJ, Sudhir K, Chou TM, Sieves RE, Zhu BQ, Sun YP, Deedwania PC, Glantz SA, Parmley WW & Chatterjee K 1997 Testosterone worsens endothelial dysfunction associated with hypercholesterolemia and environmental tobacco smoke exposure in male rabbit aorta. *Journal of American College of Cardiology* **29** 800–807.
- Jones RD, Jones HT & Channer KS 2004 The influence of testosterone upon vascular reactivity. *European Journal of Endocrinology* **151** 29–37.
- Kobayashi E, Nakano H, Morimoto M & Tamaoki T 1989 Calphostin C (UCN-1028C), a novel microbial compound is a highly potent and specific inhibitor of protein kinase C. *Biochemical and Biophysical Research Communications* **159** 548–553.
- Li H, Oehrlin SA, Wallerath T, Ihrig-Biedert I, Wohlfart P, Ulshofer T, Jessen T, Herget T, Forstermann U & Kleinert H 1998 Activation of protein kinase C α and/or epsilon enhances transcription of the human endothelial nitric oxide synthase gene. *Molecular Pharmacology* **53** 630–637.
- Li H, Herget SM, Schafer SC, Brausch I, Yao Y, Huang Q, Mang C, Lehr HA & Forstermann U 2005 Midostaurin upregulates eNOS gene expression and preserves eNOS function in the microcirculation of the mouse. *Nitric Oxide: Biology and Chemistry* **12** 231–236.

- Littleton-Kearney M & Hurn PD 2004 Testosterone as a modulator of vascular behavior. *Biological Research for Nursing* **5** 276–285.
- Liu D & Dillon JS 2002 Dehydroepiandrosterone activates endothelial cell nitric oxide synthase by a specific plasma membrane receptor coupled to G alpha (i2,3). *Journal of Biological Chemistry* **277** 21379–21388.
- Liu PY, Death AK & Handelsman DJ 2003 Androgens and cardiovascular disease. *Endocrine Review* **24** 313–340.
- Martín MC, Balfagón G, Minoves N, Blanco-Rivero J & Ferrer M 2005 Androgen deprivation increases neuronal nitric oxide metabolism and its vasodilator effect in rat mesenteric arteries. *Nitric Oxide: Biology and Chemistry* **12** 163–176.
- Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marme D & Schachtele C 1993 Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *Journal of Biological Chemistry* **268** 9194–9197.
- McNeill AM, Kim N, Duckles SP, Krause DN & Kontos HA 1999 Chronic estrogen treatment increases levels of endothelial nitric oxide synthase protein in rat cerebral microvessels. *Stroke* **30** 2186–2190.
- Michell BJ, Chen ZP, Tiganis T, Stapleton D, Katsis F, Power DA, Sin AT & Kemp BE 2001 Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. *Journal of Biological Chemistry* **276** 17625–17628.
- Mukherjee S, Coaxum SD, Maleque M & Das SK 2001 Effects of oxidized low density lipoprotein on nitric oxide synthetase and protein kinase C activities in bovine endothelial cells. *Cellular and Molecular Biology* **47** 1051–1058.
- Nielsen KC & Owman C 1971 Contractile response and amine receptor mechanism in isolated middle cerebral artery of the cat. *Brain Research* **27** 25–32.
- Nishizuka Y 1984 The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* **308** 693–698.
- Orshal JM & Khalil RA 2004 Gender, sex hormones and vascular tone. *American Journal of Physiology* **286** R233–R249.
- Perusquia M 2003 Androgen-induced vasorelaxation: a potential vascular protective effect. *Experimental and Clinical Endocrinology and Diabetes* **111** 55–59.
- Pollock JS, Förstermann U, Mitchell JA, Warner TD, Schmindt HHHW, Nakane M & Murad F 1991 Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *PNAS* **88** 10480–10484.
- Pollock JS, Nakane M, Buttery LK, Martinez A, Springall D, Polar JM, Förstermann U & Murad F 1993 Characterization and localization of endothelial nitric oxide synthase using specific monoclonal antibodies. *American Journal of Physiology* **265** C1379–C1387.
- Polytarchou C & Papadimitriou E 2005 Antioxidants inhibit human endothelial cell functions through down-regulation of endothelial nitric oxide synthase activity. *European Journal of Pharmacology* **510** 1–38.
- Sánchez-Ferrer CF & Marín J 1990 Endothelium-derived contractile factors. *General Pharmacology* **21** 589–603.
- Sader MA & Celermajer DS 2002 Endothelial function, vascular reactivity and gender differences in the cardiovascular system. *Cardiovascular Research* **53** 579–604.
- Schmeck B, Brunsch M, Seybold J, Krull M, Eichel-Streiber C, Suttrop N & Hippenstiel S 2003 Rho protein inhibition blocks cyclooxygenase-2 expression by proinflammatory mediators in endothelial cells. *Inflammation* **27** 89–95.
- Sekiguchi F, Nakahira T, Kawata K & Sunano S 2002 Responses to endothelium-derived factors and their interaction in mesenteric arteries from Wistar-Kyoto and stroke-prone spontaneously hypertensive rats. *Clinical and Experimental Pharmacology and Physiology* **29** 1066–1075.
- Shen GX 2003 Selective protein kinase C inhibitors and their applications. *Current Drug Targets* **3** 301–307.
- Simoncini T, Mannella P, Fornari L, Varone G, Caruso A & Genazzani AR 2003 Dehydroepiandrosterone modulates endothelial nitric oxide synthesis via direct genomic and nongenomic mechanisms. *Endocrinology* **144** 3449–3455.
- Spitales M & Cantrell DA 2004 Protein kinase C and beyond. *Nature Immunology* **5** 785–790.
- Sunano S, Nakahira T, Kawata K & Sekiguchi F 2001 Factors involved in the time course of response to acetylcholine in mesenteric arteries from spontaneously hypertensive rats. *European Journal of Pharmacology* **423** 47–55.
- Teoh H, Quan A & Man RY 2000 Acute impairment of relaxation by low levels of testosterone in porcine coronary arteries. *Cardiovascular Research* **45** 1010–1018.
- Tep-areenan P, Kendall DA & Randal MD 2003 Mechanisms of vasorelaxation to testosterone in the rat aorta. *European Journal of Pharmacology* **465** 125–132.
- Vanhoutte PM 1996 Endothelium-dependent responses in congestive heart failure. *Journal of Molecular and Cellular Cardiology* **28** 2233–2240.
- Ward JPT, Knock GA, Snetkov VA & Aaronson PI 2004 Protein kinase in vascular smooth muscle tone – role in the pulmonary vasculature and hypoxic pulmonary vasoconstriction. *Pharmacology and Therapeutics* **104** 207–231.
- Wedgwood S, Bekker JM & Black SM 2001 Shear stress regulation of endothelial NOS in fetal pulmonary arterial endothelial cells involves PKC. *American Journal of Physiology* **281** L490–L498.
- Wynne FL & Khalil RA 2003 Testosterone and coronary vascular tone: implications in coronary artery disease. *Journal of Endocrinological Investigation* **26** 181–186.
- Yakubu MA, Sofola OA, Igbo I & Oyekun AO 2004 Link between free radicals and protein kinase C in glucose-induced alteration of vascular dilation. *Life Sciences* **75** 2921–2932.
- Yorek MA, Coppey LJ, Gellett JS, Davison EP, Bing X, Lund DD & Dillon JS 2002 Effect of treatment of diabetic rats with dehydroepiandrosterone on vascular and neural function. *American Journal of Physiology* **283** E1067–E1075.

Received in final form 27 September 2006

Accepted 10 October 2006

Made available online as an Accepted Preprint
18 October 2006