Orchidectomy increases the formation of non-endothelial thromboxane A₂ and modulates its role in the electrical field stimulation-induced response in rat mesenteric artery

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Abstract

The aim of this study was to analyze whether endogenous male sex hormones influence the release of thromboxane A₂ (TXA₂) and its role in the electrical field stimulation (EFS)-induced response, as well as the mechanism involved. For this purpose, endothelium-denuded mesenteric arteries from control and orchidectomized male Sprague–Dawley rats were used to measure TXA₂ release; EFS-induced response, nitric oxide (NO), norepinephrine (NA), and prostaglandin (PG) I₂ release were also measured in the presence of the TXA₂ synthesis inhibitor furegrelate. Orchidectomy increased basal and EFS-induced TXA₂ release. Furegrelate decreased the EFS-induced contraction in arteries from control rats, but did not modify it in arteries from orchidectomized rats. The EFS-induced neuronal NO release and vasodilator response were increased by furegrelate in arteries from control rats, but were not modified in arteries from orchidectomized rats. Furegrelate did not modify the EFS-induced NA release or vasoconstrictor response in arteries from either control or orchidectomized rats. The EFS-induced PG I₂ release was not modified by furegrelate in arteries from control rats, but was increased in arteries from orchidectomized rats. The results of the present study show that endogenous male sex hormone deprivation i) increases non-endothelial TXA₂ release and ii) regulates the effect of endogenous TXA₂ on the EFS-induced response through different mechanisms that, at the least, involve the NO and PG I₂ systems. In arteries from control rats, inhibition of TXA₂ formation decreases the EFS-induced response by increasing neuronal NO release. In arteries from orchidectomized rats, the EFS-induced response is unaltered after the inhibition of TXA₂ formation, by increasing PG I₂ release. Journal of Endocrinology (2008) 197, 371–379

Introduction

Clinical and epidemiological studies indicate the existence of gender differences in the incidence of cardiovascular disease and have established the existence of vascular protective effects of endogenous estrogens (Teede 2007). On their part, androgens have generally been associated with impaired vascular reactivity; nevertheless, recent studies have reported beneficial properties of androgens in male vascular function (Blanco–Rivero et al. 2006a,b) similar to the effects of estrogens in women (Alexandersen et al. 1999, Ng 2007). In addition, low levels of testosterone have been reported in patients with hypertension (Phillips et al. 1993), atherosclerosis (Alexandersen et al. 1996), and coronary disease (Wrancicz et al. 2005).

Vascular tone is regulated by several mechanisms in which, depending on the type of the vessel, innervation plays a more or less important role. This regulation involves the adrenergic, cholinergic, nitrergic, peptidergic, and/or sensory innervations (Vanhoutte et al. 1981, Marco et al. 1985, Kawasaki et al. 1988) that are specific to the vascular bed under consideration. Nitric oxide (NO) is an important neurotransmitter in both the central (Bredt et al. 1992) and peripheral (Marin & Balfagón 1998) nervous systems. Electrical field stimulation (EFS) has been shown to induce NO release from nitrergic nerves in rat mesenteric arteries (Marin & Balfagón 1998, Ferrer et al. 2000, Ferrer & Balfagón 2001, del Carmen Martín et al. 2005), thus producing relaxation by stimulating soluble guanylate cyclase and increasing the intracellular levels of cGMP in the smooth muscle cells of the arterial wall (Holzmann 1982, Ignarro & Kadowitz 1985). Sex hormones have been described to modulate the release and/or function of the neuronal NO in male (del Carmen Martín et al. 2005) and female (Minoves et al. 2002) rat mesenteric arteries.

Vascular tone is also regulated by prostanoids originated by arachidonic acid metabolism through the cyclooxygenase pathway (Henrion et al. 1997, Blanco–Rivero et al. 2005, Félotou & Vanhoutte 2006). One of the most studied prostanoids is thromboxane A₂ (TXA₂) that has been implicated as a mediator in diseases such as myocardial infarction, hypertension and stroke (FitzGerald et al. 1987,
Narumiya et al. 1999). We previously reported that, in mesenteric artery from comparable rats, endogenous male sex hormones modulate endothelial TXA2 production, whether in basal conditions or after stimulation with either clonidine (Blanco–Rivero et al. 2006a) or acetylcholine (Blanco–Rivero et al. 2007), without modifying the TXA2 vasoconstrictor effect. Additionally, we have also shown that endogenous male sex hormones regulate the functional involvement of endogenous TXA2 in vascular responses of aorta (Martorell et al. 2008) and mesenteric artery (Blanco–Rivero et al. 2006a, 2007).

On the other hand, while NO has been reported to modulate the prostanooid system (Salvemini et al. 1996, Laemmel et al. 2003), there have been few reports on the action of prostanooids on the NO system (Ferrer et al. 2004, Mollace et al. 2005). Moreover, regarding the specific action of TXA2 on the NO system, a decrease in inducible (Yamada et al. 2003) as well as endothelial (Miyamoto et al. 2007) NO release has been reported. However, details about the action of TXA2 on neuronal NO release in vascular tissue remain unknown.

In the light of these considerations, the present study was designed to investigate whether endogenous male sex hormones influence the release of TXA2 and the role of the latter in the EFS-induced response, as well as the mechanism involved in this response.

Materials and Methods

Animal housing and protocols

Male Sprague–Dawley rats (6 months old) were used. Animals were housed in the Animal Facility of the Universidad Autónoma de Madrid (registration number EX-021U) in accordance with directives 609/86 CEE and RD 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 seminal vesicles atrophy confirmed successful surgery. The rats were weighed and 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 endothelium was removed to eliminate the main source of vasoactive substances, including NO. This avoided possible actions on endothelial cells by different drugs that could lead to misinterpretation of results. The endothelium was removed by gently rubbing the luminal surface of the segments with a thin wooden stick. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the USA National Institutes of Health (publication no. 85-23 revised 1985).

TXA2, norepinephrine (NA), and prostaglandin I2 (PGI2) release

The production of TXA2 and PGI2 in vivo was typically monitored by measuring their stable metabolites TXB2 and 6-keto-PGF1α respectively, using a TXB2 or a 6-keto-PGF1α EIA kit (Cayman Chemical, Annator, MI, USA). NA was measured using Noradrenaline Research EIA (Labor Diagnostika Nord, Nordhom, Germany).

The endothelium-denuded rat mesenteric segments were preincubated for 30 min in 5 ml KHS at 37°C, continuously gassed with a 95% O2–5% CO2 mixture (stabilization period). After several 10-min washout periods in a bath containing 400 µl KHS, the medium was collected to measure basal release. Once the chamber was refilled, cumulative EFS periods of 30 s at 1, 2, 4, 8, and 16 Hz at 1-min intervals were applied, and the medium was collected to measure the EFS-induced release. Each assay was performed following the manufacturer’s instructions. Results were expressed as pg/ml × mg tissue for TXA2 and PGI2 release and as ng/ml × mg tissue for NA release.

Vascular reactivity

The method used for 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; Quincy, MA, USA); this was connected in turn to a model 7D Grass polygraph. For the EFS experiments, the segments were mounted between two platinum electrodes 0·5 cm apart and connected to a stimulator (Grass, model S44) modified to supply appropriate current strength. The 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. The endothelium removal did not alter the contractions elicited by 75 mM KCl. After a washout period, the absence of vascular endothelium was tested by the inability of 10 µM acetylcholine (ACh) to relax segments precontracted with 1 µM NA.

Frequency–response curves for EFS (1, 2, 4, 8, and 16 Hz) and concentration–response curves for NA (10 nM–10 µM) were obtained. The parameters used for EFS were 200 mA, 0·3 ms, and 1–16 Hz, for 30 s with an interval of 1 min between each stimulus, the time required to recover basal tone. A washout period of at least 1 h was necessary to avoid desensitization between consecutive curves. Three successive frequency–response curves separated by 1-h intervals produced similar contractile responses.

To determine the effect of endogenous TXA2 on the response induced by EFS, the TXA2 synthase inhibitor,
furegrelate (1 μM), was added to the bath 30 min before the second frequency–response curve.

To determine the possible effect of endogenous TXA₂ on the NA-induced vasoconstrictor response, furegrelate was added to the bath 30 min before performing the NA concentration–response curve. The possible effect of furegrelate on the vasodilator effect of NO was also analyzed by obtaining concentration–response curves for the NO donor sodium nitroprusside (SNP) in 30-min furegrelate preincubated arteries.

**NO release**

Endothelium-denuded 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 EFS periods of 30 s at 1, 2, 4, 8, and 16 Hz at 1-min intervals were applied. The fluorescence of the medium was measured at room temperature using a spectrofluorimeter (LS50 Perkin–Elmer instruments; FL WinLab Software) with excitation wavelength set at 495 nm and emission wavelength at 515 nm. This method has been validated by comparing the results obtained with DAF and with those obtained by nitrites measurement (del Carmen Martín et al. 2005).

The interference of endogenous TXA₂ on NO release was studied by incubating the arteries with the TXA₂ synthase inhibitor furegrelate (1 μM) 30 min before collecting medium.

Each data was calculated by subtracting the blank measures from the corresponding NO release obtained. Blank measures were collected in the same way from segment–free medium in order to subtract background emission. The specificity of the method has already been demonstrated (del Carmen Martín et al. 2005, Blanco-Rivero et al. 2006c). The amount of NO released was expressed as arbitrary units/mg tissue.

**Drugs**

L-NA hydrochloride, ACh chloride, L-NAME hydrochloride, SNP and DAF-2 were obtained from Sigma–Aldrich and furegrelate from Cayman chemical (Europe). Stock solutions (10 mM) of drugs were made in distilled water, except for NA, which was dissolved in an 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.

**Data analysis**

The responses elicited by EFS or NA were expressed as a percentage of the contraction induced by 75 mM KCl. The relaxation induced by SNP was expressed as a percentage of the initial contraction elicited by 1 μM NA. Results are given as mean ± S.E.M. Statistical analysis was done by comparing the curve obtained in the presence of the different substances with the previous or control curve by means of repeated-measures ANOVA. For the experiments on TXA₂, NO, NA, and PGI₂ release, the statistical analysis was done using Student’s t-test for unpaired experiments. P < 0.05 was considered significant.

**Results**

**Body weight**

Orchidectomy slightly decreased rat body weight (control, 469.7 ± 8.4 g; orchidectomized, 428 ± 5.4 g; n = 10; P < 0.05), but this did not affect the size of mesenteric artery.

**TXA₂ release**

Orchidectomy increased basal TXA₂ release. The EFS–induced TXA₂ release was greater in arteries from orchidectomized than control male rats (Fig. 1).

**Vascular reactivity**

Preincubation with the TXA₂ synthase inhibitor furegrelate (1 μM) did not modify basal tone in arteries from either rat group; furegrelate decreased the EFS–induced contraction in arteries from control male rats (Fig. 2a), but did not modify it in arteries from orchidectomized rats (Fig. 2b).

Preincubation with furegrelate did not modify the NA–induced contraction in arteries from control (Fig. 3a) or orchidectomized rats (Fig. 3b).

Preincubation with furegrelate increased the SNP–induced relaxation in arteries from control male rats (Fig. 4a), but did not affect that induced in arteries from orchidectomized rats (Fig. 4b).

**Figure 1** Basal and EFS–induced thromboxane A₂ (TXA₂) release in mesenteric denuded segments from (a) control and (b) orchidectomized rats. Results (mean ± S.E.M.) are expressed as pg/ml × mg tissue. Number of animals is indicated in parenthesis. *P < 0.05 compared with basal TXA₂ release. **P < 0.05 compared with the respective control.
Basal and EFS-induced NO release was similar in mesenteric arteries from both control and orchidectomized male rats (ANOVA, $P > 0.05$). In segments from control male rats, preincubation with the TXA2 synthase inhibitor, furegrelate, did not modify the basal NO release, but did increase the EFS-induced NO release (Fig. 5a). By contrast, in segments from orchidectomized rats, preincubation with furegrelate did not affect the basal or EFS-induced NO release (Fig. 5b); similar results were obtained when the furegrelate concentration was increased to 10 μM (Fig. 5b).

**NA release**

Basal and EFS-induced NA release was similar in mesenteric arteries from both control and orchidectomized male rats (ANOVA, $P > 0.05$), as already reported using the tritium release method for measuring NA release (Blanco-Rivero et al. 2006c). The presence of furegrelate did not modify either the basal or the EFS-induced NA release in arteries from control (Fig. 6a) or orchidectomized (Fig. 6b) rats.

**PGI2 release**

Basal and EFS-induced PGI2 release was similar in mesenteric arteries from both control and orchidectomized male rats (ANOVA, $P > 0.05$). Furegrelate did not modify this release in arteries from control rats (Fig. 7a), but increased it in arteries from orchidectomized rats (Fig. 7b).

**Discussion**

Recent studies have reported beneficial effects of androgens in cardiovascular function in males through different mechanisms that involve NO and prostanoids (Jones et al. 2004, Martorell et al. 2008). The activation of androgen receptors, expressed in both the endothelial (Yu et al. 2007) and smooth muscle (Ma et al. 2005) cells, regulates cell signaling pathways. However, the vascular effects of male sex hormones have also been reported to be independent of androgen receptor activation (Liu et al. 2008) and to interact
with intracellular calcium regulatory mechanisms, as reported for other sex steroids hormones (Zhang et al. 2006).

The role of NO and prostanoids in regulating vascular tone is well established (Henrion et al. 1997, Ferrer & Osol 1998, Busse & Fleming 2003), and interaction between these two systems has been described, particularly that of NO acting on prostanoids release (Laemmel et al. 2003, Mollace et al. 2005); however, reports describing the action of prostanoids on the NO system are scarce (Ferrer et al. 2004). TXA2 is one of the most important vasoconstrictor prostanoids with stimulatory action on proliferation or hypertrophy of vascular smooth muscle cells (Hanasaki et al. 1990), and is implicated as a mediator in diseases such as myocardial infarction, hypertension, and stroke (FitzGerald et al. 1987, Narumiya et al. 1999). We previously reported that endothelial TXA2 release in mesenteric artery (Blanco-Rivero et al. 2006a, 2007) and aorta (Martorell et al. 2008) was increased in arteries from orchidectomized rats. The fact that the levels of testosterone dramatically decreased in orchidectomized rats (Martorell et al. 2008) seems to indicate that the vascular effects observed are testosterone dependent. However, the involvement of hormones and/or gonadal factors other than testosterone cannot be ruled out. Thus, vascular endothelial growth factor, basic fibroblast growth factor, transforming growth factor-β, or hyalurondiase with gonadal origin (Lissbrant et al. 2003) could all play an important role in vascular function (Rahmanian & Heldin 2002).

Therefore, we studied the possible modification of EFS-induced TXA2 release by endogenous male sex hormones. We found that orchidectomy increased the basal TXA2 release, as previously reported in endothelium intact mesenteric arteries from comparable animals (Blanco-Rivero et al. 2006a, 2007); it is important to mention that basal release in arteries without endothelium was lower than that in arteries with intact endothelium, confirming endothelial and smooth muscle cells as sources of TXA2 production.

We have previously demonstrated that EFS induced similar contractile responses in mesenteric arteries from control and orchidectomized rats, responses that appear to be mediated by NA release from adrenergic nerve terminals and the

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**Figure 4** Effect of furegrelate on the concentration-dependent curves for sodium nitroprusside in mesenteric artery segments from (a) control and (b) orchidectomized rats. Results (mean ± S.E.M.) are expressed as percentage of inhibition of contraction induced by 1 μM NA. Number of animals is indicated in parenthesis.

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**Figure 5** Effect of furegrelate (Fure.) on the basal and EFS-induced NO release in mesenteric denuded segments from (a) control and (b) orchidectomized rats. Results (means ± S.E.M.) are expressed as arbitrary units/mg tissue. Number of animals is indicated in parenthesis. *P < 0.05 compared with basal TXA2 release. **P < 0.05 compared with the respective control.
subsequent activation of α-adrenoceptors (del Carmen Martín et al. 2005); in addition, we found that the contractile response to exogenous NA was decreased by orchidectomy (del Carmen Martín et al. 2005), suggesting that EFS could increase NA release in arteries from orchidectomized rats; however, we later demonstrated that the EFS-induced NA release was not modified by orchidectomy (Blanco-Rivero et al. 2006c), which indicates that other vasoconstrictor factors could be released when the artery was electrically stimulated. Since the EFS-induced release of TXA2 has been demonstrated in hypertensive rats (Aras-López et al. 2007), we analyzed the EFS-induced TXA2 release in normotensive rats, as well as the possible role of endogenous male sex hormones in that release. We observed that EFS induced a greater TXA2 formation in arteries from orchidectomized than control rats, which is in line with reports showing increased TXA2 release after activation of different receptors (Blanco-Rivero et al. 2006a, 2007). This result also indicates that TXA2 could be the contractile factor that is released when the artery is electrically stimulated, as suggested previously (Blanco-Rivero et al. 2006c). Increased TXA2 release would explain the non-modification of the EFS-induced response in arteries from control and orchidectomized rats, in spite of the fact that the NA response was diminished in arteries from the latter animals.

The next step was to analyze the function of endogenous TXA2 in the EFS-induced response, as well as the dependence on male sex hormones. Preincubation with furegrelate did not modify the basal tone in arteries from control or orchidectomized rats, indicating that endogenous TXA2 does not have a direct effect on vascular tone regulation in basal conditions. We showed that furegrelate decreased the EFS-induced response in arteries from control rats, but did not modify it in arteries from orchidectomized rats, indicating that the effect of endogenous TXA2 on the EFS response is under male sex hormone regulation.

It is widely reported that mesenteric arteries possess nitrergic (Marín & Balfagón 1998, del Carmen Martín et al. 2005), sympathetic (Li & Duckles 1992), and sensory (Kawasaki et al. 1988) innervations that modulate vasomotor tone; therefore, the EFS-induced contraction is the result of a balance between opposing vasoconstrictor and vasodilator factors (Vanhoutte 1988).

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**Figure 6** Effect of furegrelate on the basal and EFS-induced norepinephrine (NA) release in mesenteric denuded segments from (a) control and (b) orchidectomized rats. Results (means ± S.E.M.) are expressed as ng/ml × mg tissue. Number of animals is indicated in parenthesis. *P<0.05 compared with basal NA release. †P<0.05 compared with the respective control.

**Figure 7** Effect of furegrelate on the basal and EFS-induced PGI2 release in mesenteric denuded segments from (a) control and (b) orchidectomized rats. Results (means ± S.E.M.) are expressed as pg/ml × mg tissue. Number of animals is indicated in parenthesis. *P<0.05 compared with basal PGI2 release. †P<0.05 compared with the respective control.
induced by the NO donor, SNP, was also increased by vasomotor response, as reported previously (Molderings et al. 1998, Hoang et al. 2003), indicates that furegrelate did not modify either NA release or its function of neuronal NO, as well as the dependence on endogenous male sex hormones. We found that in arteries from control rats, furegrelate increased the neuronal NO release, which is in line with reports describing an inhibitory effect of TXA2 on inducible (Yamada et al. 2003) and endothelial (Miyamoto et al. 2007) NO release. The vasodilator response induced by the NO donor, SNP, was also increased by furegrelate, showing that endogenous TXA2 negatively modulates both the release and the vasodilator effect of neuronal NO. These results could also explain the decreased EFS-induced contraction in the presence of furegrelate, but alterations in the release and function of neurotransmitters other than NO cannot be ruled out.

Different modulating effects of TXA2 on NA release have been reported, including inhibition (Nishihara et al. 2000) and non-modification (Rump & Schollmeyer 1989). The fact that furegrelate did not modify either NA release or its vasomotor response, as reported previously (Molderings et al. 1998, Hoang et al. 2003), indicates that endogenous TXA2 does not alter the function of sympathetic innervation in arteries from control rats.

By contrast, in arteries from orchidectomized rats, furegrelate did not modify the basal and EFS-induced NO release; since TXA2 formation was greater in arteries from orchidectomized than control rats, we used a higher concentration of furegrelate, and still obtained similar results. In addition, the vasodilator response induced by SNP was not modified by furegrelate. These results show that endogenous TXA2 does not regulate the release or function of neuronal NO in arteries from orchidectomized rats, in contrast to what occurs in arteries from control rats.

Regarding noradrenergic neurotransmission, we found that furegrelate modified neither the NA release nor the vasoconstrictor response induced by exogenous NA, indicating that the function of the sympathetic innervation is not regulated by endogenous TXA2 in arteries from orchidectomized rats, as was also observed in arteries from control rats.

Since TXA2 was higher in arteries from orchidectomized rats, and since endogenous TXA2 did not modify either the release or function of NO or NA, the unaltered EFS-induced response observed in the presence of furegrelate could be explained through the release of vasodilator factors that would counterbalance the vasoconstrictor effect of TXA2.

One of the more plausible candidates would be PGI2, since crosstalk between TXA2 and PGI2 systems has been reported (Cheng et al. 2002, Martorell et al. 2008) and joint increases in PGI2 and TXA2 synthesis have been shown in pathological conditions (FitzGerald 1991, Caughey et al. 2001). Therefore, we measured the production of PGI2 in the presence of furegrelate in mesenteric arteries from both control and orchidectomized rats. First, we found that orchidectomy did not modify either the basal or EFS-induced PGI2 release, in contrast to the increased PGI2 formation observed in aorta from comparable animals (Martorell et al. 2008); these results are in line with reports showing smooth muscle (Wang et al. 1993, Ferrer et al. 2004) and/or neuronal (Snitsarev et al. 2005) cells as cellular sources of PGI2. Concerning the effect of endogenous TXA2 on PGI2 production, we observed that the inhibition of the endogenous TXA2 synthesis did not modify the release of PGI2 in arteries from control rats, although it did increase PGI2 release in arteries from orchidectomized rats. This result reinforced the suggestion that the possible increase in the release of vasodilator substances that counterbalance the vasoconstrictor effect of TXA2, although the involvement of substances other than PGI2 cannot be ruled out.

In summary, this study demonstrates that non-endothelial TXA2 release is increased in mesenteric arteries from orchidectomized rats. The effect of endogenous TXA2 on the EFS-induced response is also regulated in a gonad-dependent way, suggesting that male sex hormones modulate different simultaneous cell signaling pathways. In arteries from control rats, inhibition of TXA2 formation decreases the EFS-induced response by increasing neuronal NO release, but in arteries from orchidectomized rats, the EFS-induced response is unaltered after the inhibition of TXA2 formation, by increasing PGI2 release.

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