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Androgens and Vascular Function

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1. Introduction

It is widely recognized that vascular function is modulated by the endocrine system. So it is known that hormones as aldosterone, renin-angiotensin II system, thyroids hormones, oxitocine, ghrelin, vasopressine, etc exert action on the vascular tone (Axelband *et al*., 2011; Nguyen & Touyz, 2011; Szmydynger-Chodobska *et al*., 2011; Tesauro *et al*., 2010;). Regarding sex hormones, epidemiological studies have demonstrated that there is a gender difference in the morbidity associated with hypertension and that there is an increased prevalence of cardiovascular diseases in postmenopausal women (Leung *et al*., 2007; Teede, 2007). Simultaneously, androgens had been associated with an increased risk of cardiovascular disease, but recent studies have explored protective effects of androgens in males (Jones, 2010; Traish and Kypreos, 2011). For example, it has been demonstrated that men with coronary artery disease have decreased levels of testosterone, which were conversely correlated to the degree of coronary artery narrowing (Saad *et al*., 2008). Likewise, lower testosterone, predicted incident stroke and transient ischemic attack in older men (Braga-Basaria, 2006). In general terms, it seems to be demonstrated that men with cardiovascular disease had lower levels of testosterone, and what is more important, new emerging evidence points to androgen deficiency more likely to be associated with cardiovascular diseases than gender per se (Traish & Kypreos, 2011). Testosterone deficiency alters carbohydrate, lipid and protein metabolism, this contributing to oxidative stress, endothelial dysfunction and increased production of pro-inflammatory factors, promoting alterations on vascular function. Among the alterations induced by testosterone deficiency are the loss of muscle mass and strength, increasing visceral fat mass, reduced libido, erectile dysfunction, increased osteoporosis, lethargy, lack of energy, and changes in mood. In addition, testosterone deficiency has been associated with increased risk of metabolic syndrome, type 2 diabetes, obesity, insulin resistance and atherosclerosis (Jones & Saad, 2009; Kapoor *et al*., 2005).

Most of results obtained about that effects have been obtained from patients with prostate cancer subjected to androgen deprivation therapy. This therapy improves cancer related symptoms and quality of life (Bain, 2010), but shows side effects as sexual dysfunction, decreased lean body mass, decreased quality of life, osteoporosis, and detrimental changes in metabolic status (Basaria & Dobs, 2001; Chodak *et al*., 2002; Smith *et al*., 2002). Increased insulin levels and insulin resistance and increased prevalence of fasting hyperglycemia and hypertriglyceridemia have also been observed in men with prostate cancer treated with

androgen deprivation therapy (Braga-Bassaria *et al*., 2006; Keating *et al*., 2006). This type of therapy provides an invaluable method to correlate vascular alteration and sex hormone status. However, at mechanistic level the animal models are of valuable interest. In this sense our research group has been focused on analyzing the effects produced by the loss of gonadal function on vascular reactivity, as well as some of the underlaying mechanisms.

Vascular tone is regulated by several mechanisms in which nitric oxide (NO) plays an important role. NO is formed through several NO synthases (NOS), i.e., endothelial NOS (eNOS), inducible (iNOS), and neuronal (nNOS). Independently from the source of NO, one of the major downstream events occurring after NO release is an increase in cGMP formation through soluble guanylate cyclase stimulation, and the subsequent activation of cGMP-dependent protein kinase (PKG) (Murad, 1997). PKG contributes to reduce the intracellular calcium concentration through a wide spectrum of PKG substrates, leading to vasodilation (Lincoln *et al*., 2001; Munzel *et al*., 2003). In addition, NO (Bolotina *et al*., 1994) and cGMP (Ferrer *et al*., 1995) can induce membrane hyperpolarization and subsequent relaxation.

Concerning NO, and independently from the NO-activated signalling pathway, it is important to take into account that vascular function of endothelial NO depends on its bioavailability, which is determined by the rate of NO production and by its scavenging by superoxide anion. Therefore, the elimination of superoxide anion within the vessel wall is fundamental and it is performed by superoxide dismutases (SODs) (Wolin, 2002). It is well understood that alterations of different steps along the NO pathway determine its effect on the vascular tone.

Vascular tone is also regulated by prostanoids originated by arachidonic acid metabolism through the cyclooxygenase (COX) pathway (Henrion *et al*., 1997). One of the best studied prostanoids is thromboxane A_2 (TXA_2) which has been implicated as mediator in diseases such as myocardial infarction and hypertension (FitzGerald et al., 1987). Additionally, the role of COX derivatives other than TXA_2, such as prostaglandin (PG) $F_{2\alpha}$ and PGE_2, or PGI_2 which can induce a vasoconstrictor or vasodilator response is the subject of numerous studies (Blanco-Rivero *et al*., 2005; Félétou & Vanhoutte, 2006) since they could also participate in vascular dysfunction.

On the other hand, proteins kinases are important regulators of different cell signalling pathways. Among different proteins kinases, PKC merits special attention since it is able to regulate the activity of different enzymes such as NOS and COX-2 (Kim *et al*., 2008; Shanmugam *et al*., 2004). In turn, PKC can be activated by different reactive oxygen species. At this point, it is important to note that sex hormones possesses antioxidant properties, and the loss of gonadal function can induce oxidative stress and, in turn triggers modulatory actions in different cell signalling pathways that are working simultaneously to ensure the optimal response of the vessel to different stimuli.

For this, this chapter will review how the loss of gonadal function modifies the release and function of different mediators, such as reactive oxygen species, nitric oxide and prostanoids, and their functional involvement in the reactivity of aorta and mesenteric artery of the male rats. Future studies in the research field of androgens on cell signaling pathways will be commented, since they will be of important interest to implement therapeutic strategies that could improve vascular function.

2. Androgens and nitric oxide

The functional role of NO in vascular tone regulation has been widely reported (Furchgott & Zawadzki, 1980; Toda & Okamura, 2003; Vanhoutte 1996). NO is formed through several

NO synthases (NOS), i.e, endothelial NOS (eNOS), inducible (iNOS) and neuronal (nNOS) (Förstermann *et al*., 1991). Most of the studies about the effects of androgens on vascular function have been focused on analyzing the interaction between androgens and endothelial NO (Tep-areenan *et al*., 20043; Jones *et al*., 2004). More specifically, most studies have been focused on analyzing the effects of androgenic derivatives on different aspects of the NO system, such us eNOS expression, NO release or NO vasodilator effect (Ceballos *et al*., 1999; Hutchison *et al*., 1997; Teoh *et al*., 2000). However, few studies exist about the specific effect of endogenous male sex hormones on these aspects referred to endothelial or neuronal NO when they are simultaneously studied. Our data were obtained from aorta and mesenteric artery from intact and orchidectomized male Sprague-Dawley rats (6 months old), and seem not to be related to haemodynamic changes, since orchidectomy did not modify blood pressure (137 $\pm$ 5.8 mm Hg in control rats and of 145 $\pm$ 6.2 mm Hg in orchidectomized rats; $p > 0.05$). Moreover, the results on vascular function would have to be androgen-related, as confirmed by the decreased testosterone levels (control: 2404 $\pm$ 323 pg/mL; orchidectomized: 220 $\pm$ 49 pg/mL; n = 6; $p < 0.001$).

The reported effects of androgens on NO release are contradictory. Testosterone impairs relaxation and worsens endothelial dysfunction in male rabbits (Hutchison *et al*., 1997). However, it has also been reported that testosterone or its derivatives increase eNOS (Liu & Dillon, 2002; Weiner *et al*., 1994) and nNOS activity in the central nervous system of guinea pig (Weiner *et al*., 1994) and mouse (Scordalakes *et al*., 2002). Nevertheless, to our knowledge, we showed first evidence on the effect of endogenous male sex hormones on the nNOS/eNOS expression and nNOS-/eNOS-derived NO release in vascular tissues (Martin et al., 2005; Blanco-Rivero et al., 2007).

2.1 Neuronal nitric oxide

The analysis of the expression of nNOS, by Western blot, showed that it was higher in segments from control rats than from orchidectomized rats (Martín *et al*., 2005). The NO release was quantified by measuring nitrite production and also the fluorescence emitted by DAF-2, and the results showed that electrical field stimulation EFS-induced NO release was similar in segments from both control and orchidectomized rats. The measured NO release seems to come from nerve endings, since preincubation with tetrodotoxin abolished the nitrite release in endothelial-denuded segments from both groups of rats. This result indicates that male sex hormones apparently do not modulate nNOS-derived NO release, in contrast to our observations with female sex hormones in which we found that the loss of these hormones provoked by ovariectomy increased nNOS-derived NO release (Minoves *et al*., 2002).

To study the involvement of neuronal or endothelial NO on vasomotor responses, EFS induced contractile responses in endothelium-denuded mesenteric segments were analyzed by using vascular reactivity technique (Nielsen & Owman, 1971). The contractile response induced by EFS were practically abolished by tetrodotoxin and markedly reduced by phentolamine, the respective blockers for nerve impulse propagation and α-adrenoceptors in arteries from both control and orchidectomized rats. Therefore, these responses appear to be mediated by noradrenaline (NA) release from adrenergic nerve terminals and the subsequent activation of α-adrenoceptors in both experimental hormonal conditions, as has been described in other rat strains (Ferrer *et al*., 2000; 2001).

The present results show that vasoconstrictor response to KCl and exogenous NA are diminished in segments from orchidectomized male rats, which is in agreement with most studies demonstrating that testosterone treatment enhanced the action of several contractile

agents (Baker *et al*., 1978; Calderone *et al*., 2002; Greenberg *et al*, 1974). However, the responses induced by EFS was similar in both groups of rats Fig. 1 which would suggest that EFS increased the release of a vasoconstrictor factor, that could be the NA release from adrenergic endings in segments from orchidectomized animals.

Concerning this point, most studies have been performed in the central nervous system and, conflicting results exist. Thus, a decrease (Guan &Dluzen, 1991; Holmquist *et al*., 1994), an increase (Shan & Dluzen, 2002; Siddiqui & Shah, 1997) and no modification (Agostini *et al*., 1981; Chen *et al*., 1999) of NA release have been reported in orchidectomized male animals. In a later study we demonstrated that the NA release was not modified by orchidectomy (Blanco-Rivero *et al*., 2006).

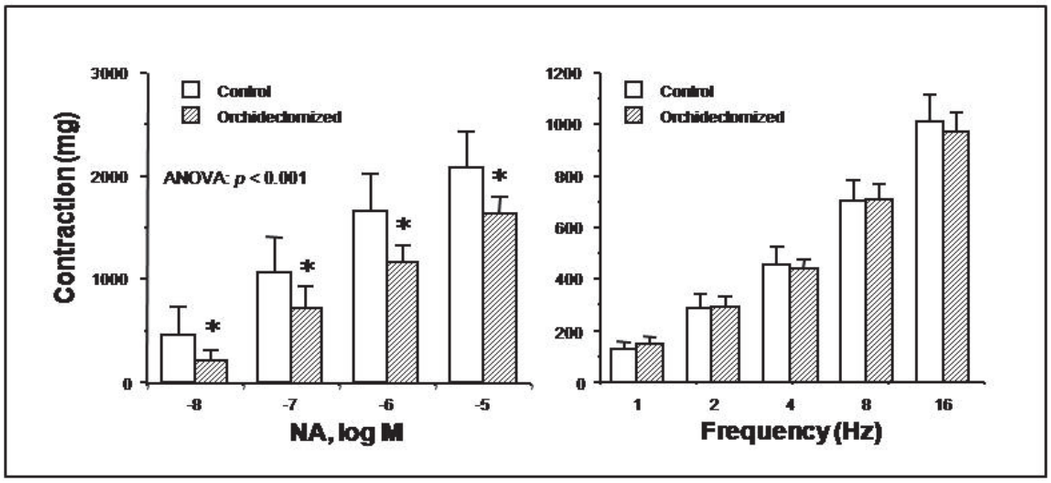

Fig. 1. Contractile response induced by cumulative concentration-response curves to noradrenaline (NA) and by frequency-response curves in denuded mesenteric arteries from control and orchidectomized rats. Results (means ± SEM) are expressed in milligram. Number of animals: 25-30. * p<0.01 compared with control rats (from Martín et al., 2005).

Since sex hormone modulation of the calcitonin gene related peptide (CGRP) system has been described (Sun *et al*., 2001), and that the CGRP, the essential neurotransmitter in sensory nerves (Kawasaki *et al*., 1988), had been proposed to play a role in vascular tone regulation, the participation of sensory innervation in the vasomotor response to EFS was assessed by the use of capsaicin, which selectively depletes the sensory nerves of the neurotransmitter (Li & Duckles, 1992). Capsaicin did not have a significant effect on the vasomotor response to either EFS or exogenous NA in mesenteric arteries from control and orchidectomized rats (data not shown), indicating that sensory innervation does not modulate the vasomotor response to EFS in our experimental conditions. Sun et al., (2001) analyzed how orchidectomy modulates CGRP release, while we analyzed the functional involvement of the sensory innervation in the response to EFS without separately studying CGRP release and/or response.

The NOS inhibitor L-NAME increased the vasoconstrictor response to EFS in segments from both control and orchidectomized rats. The fact that the endothelium was removed and that AMT, an inhibitor of iNOS (Ferrer *et al*., 2000; Ishikawa & Quock, 2003; Tracey *et al*., 1995), did not modify the response induced by EFS in segments from both rat groups, reinforced the neuronal origin for the NO release.

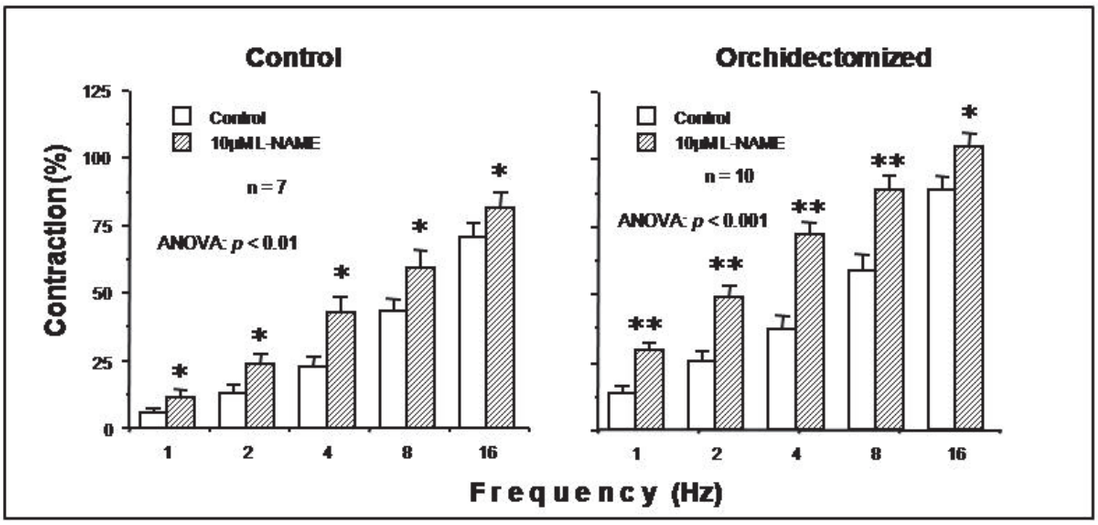

Fig. 2. Effect of L-NAME on the frequency-response curves performed in mesenteric arteries from control and orqchidectomized rats. Results are expressed as a percentage of a previous tone with 75 mM KCl. n, number of animals; *p<0.05; **p<0.01 compared with control rats (from Martín et al., 2005).

The greater response to EFS in the presence of L-NAME in segments from orchidectomized animals compared with the response obtained in segments from control rats Fig. 2 indicated differences in steps downstream from neuronal NO release, that will be considered later.

The reported effects of androgens on NO release are contradictory. Testosterone impairs relaxation and worsens endothelial dysfunction in male rabbits (Hutchison *et al*., 1997). However, it has also been reported that testosterone or its derivatives increase eNOS (Liu & Dillon, 2002; Weiner *et al*., 1994) and nNOS activity in the central nervous system of guinea pig (Weiner *et al*., 1994) and mouse (Scordalakes *et al*., 2002). Nevertheless, to our knowledge, there is no experimental evidence on the effect of male sex hormones on the nNOS expression and nNOS-derived NO release in vascular tissues. Therefore, we analysed the expression of nNOS by western blot, and found it was higher in segments from control rats than from orchidectomized rats. We also quantified the NO release induced by EFS in segments from both groups of rats by measuring nitrite production and the fluorescence emitted by DAF-2, and the results showed that the EFS-induced NO release was similar in segments from both control and orchidectomized rats.

2.2 Endothelial nitric oxide

The eNOS expression in aorta and mesenteric artery showed that not was modified by orchidectomy (Blanco-Rivero *et al*., 2007; Martorell *et al*., 2008), in contrast to that observed in endothelial-denuded mesenteric artery (Martín *et al*., 2005), which indicates that endogenous male sex hormones act in a different manner depending on the target protein. Additionally, these results also indicate that effects induced by endogenous hormones are quite different from those induced by exogenous hormones, since 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 increase (Liu & Dillon, 2002; Simoncini *et al*., 2003) and decrease (Mukherjee *et al*., 2001) have both been reported. Others researchers have

demonstrated that androgens increased (Orshal & Khalil, 2004; Wynne & Khalil, 2003) and decreased (Ba *et al*., 2001; Gonzales *et al*., 2004) the vasodilator effect of endothelial NO. In our experimental rat model, we have found that the release of endothelial NO was not modified by orchidectomy, either in mesenteric artery (Blanco-Rivero *et al*., 2007) or aorta (Blanco-Rivero *et al*., 2006). However, orchidectomy reduced the endothelium-dependent vasodilator response induced by Acetylcholine (Ach) in mesenteric artery while increased in rat aorta (Fig. 3).

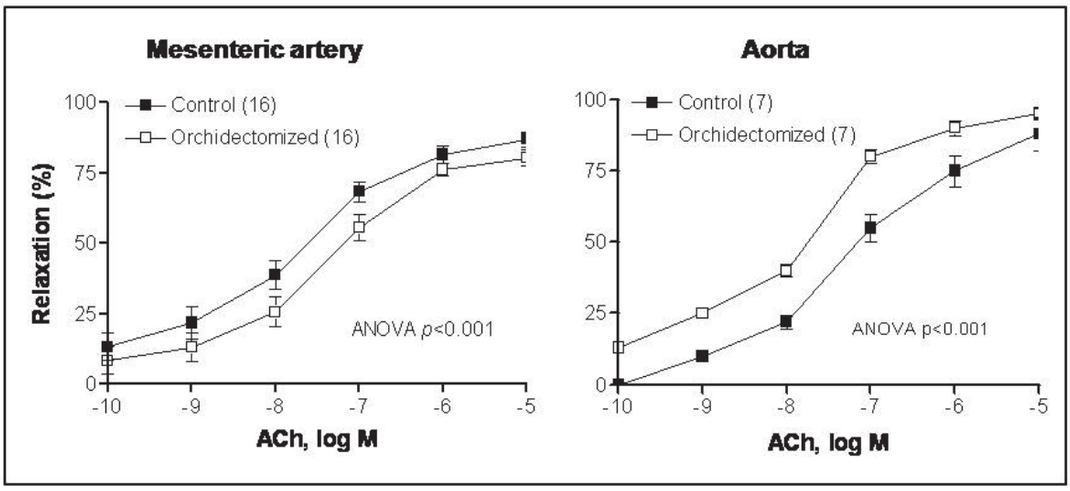

Fig. 3. Concentration-response curve to acetylcholine (ACh) in mesenteric artery and aortic segments from control and orchidectomized rats. Results are expressed as a percentage of a previous tone with noradrenaline. Number of animals is indicated in parentheses (from Blanco-Rivero *et al*., 2007; Ferrer *et al*., 1999).

These results could indicate that orchidectomy, depending on the vessel, may alter the sensitivity to NO and/or the release of factors other than NO, that will be revised in the next sections.

3. Androgens and production of reactive oxygen species

Reactive oxygen species are involved in metabolising NO (Gryglewski *et al*. 1986, Ferrer *et al*. 2000, 2001) and it has been reported that can induce oxidative processes associated with cardiovascular disorders (Harrison 1994; Munzel *et al*., 1997). 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). Therefore, both production and removal of superoxide anion are important contributors to the maintenance of appropriated levels of this oxygen species. Since several studies have demonstrated androgens antioxidants properties of androgens (Békési *et al*., 2000; Yorek *et al*., 2002), the effect of the orchidectomy on the production of superoxide anion was analyzed, by measuring the lucigenin chemiluminescence (control =117 $\pm$ 43 U/mg/min, n= 5; orchidectomized= 546 $\pm$ 58 U/mg/min, n= 5; $p < 0.001$). A similar result was obtained with acetylcholine-induced superoxide anion in aortas from the same animals (control: 34.7 $\pm$5.6 U/mg/ min, n= 4; orchidectomized: 132.8 $\pm$ 3.2, n= 4; $p< 0.001$). These results agree with previous studies showing the antioxidants properties of androgens (Békési *et al*., 2000; Yorek *et al*., 2002). The elimination of superoxide anion within the vessels is fundamental since this oxygen specie can reduce the NO bioavailability.

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/ZnSOD, mitochondrial MnSOD, and extracellular ecSOD –which is also Cu/Zn-dependent (Strehlow *et al.*, 2003), we have focused on analyzing the participation of Cu/ZnSOD 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). We analyzed the expression and activity of SODs because there was no information about modulation of these enzymes by endogenous male sex hormones. We found that both the expression and activity of Cu/ZnSOD were increased in aortic segments from orchidectomized rats (Fig. 4). These results were in line with other studies that described an increased expression and/or activity of SOD in cardiovascular pathologies, in which superoxide anion overproduction exists (Kobayashi *et al.*, 2002, Tanaka *et al.*, 2005).

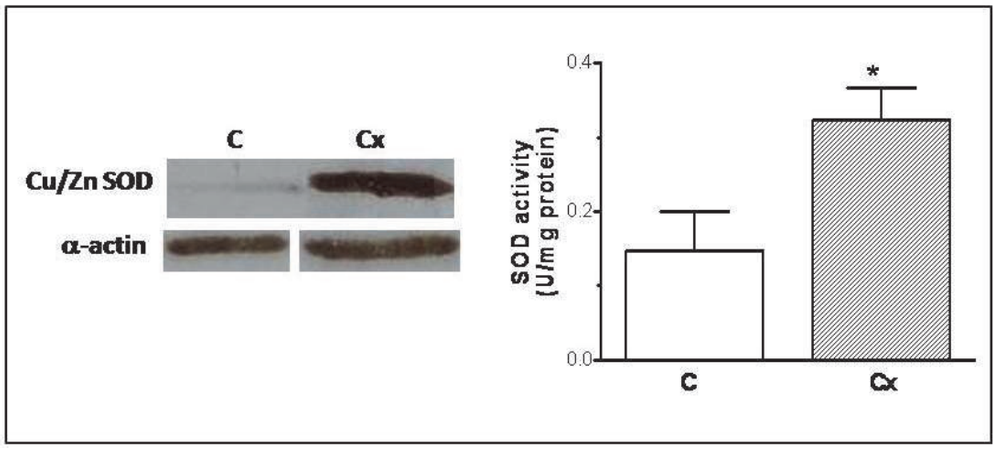

Fig. 4. Representative western blot for Cu/ZnSOD and) α-actin expression and SOD activity in aortic segments from control (C) and from orchidectomized (Cx) rats (from Blanco-Rivero *et al*., 2006).

In addition, these results indicated that the increases in both expression and activity of Cu/ZnSOD could be a compensatory mechanism to eliminate the elevated superoxide anion formation induced by orchidectomy in male rats. On the other hand, additional mechanisms may exist to try to maintain vasodilator function. One of these mechanisms could be the activation of calcium-dependent potassium channels by superoxide anion (Ferrer *et al.*, 1999). In that report, we confirmed that the calcium dependent potassium channels (K_{Ca}) activation was specifically induced by superoxide anion, since the NO synthesis was inhibited.

However, the vascular response depends on the vascular bed analyzed. For example, in mesenteric artery from orchidectomized rats, the vascular ROS-induced relaxation was due to the production of peroxynitrite and hydrogen peroxide H_2O_2. The increased formation of peroxynitrite in arteries from orchidectomized rats was studied by immunohistochemical localization and was reinforced by functional analysis on vasodilator response induced by sodium nitroprusside (SNP):

The presence of SOD increased the response to SNP in segments from orchidectomized rats which could be mediated through the decreased peroxynitrite formation by removing superoxide anion, and the simultaneous increased of H_2O_2 formation, which exert

vasodilator action (Martín *et al*., 2005; Rubanyi & Vanhoutte, 1986; Wei *et al*., 1996). The simultaneous incubation of arteries with SOD and catalase reversed the vasodilator response to SNP, indicating that the H_2O_2 synthesized in the presence of SOD, would participate in that vasodilator effect, when the formation of peroxynitrite is inhibited (Fig. 5).

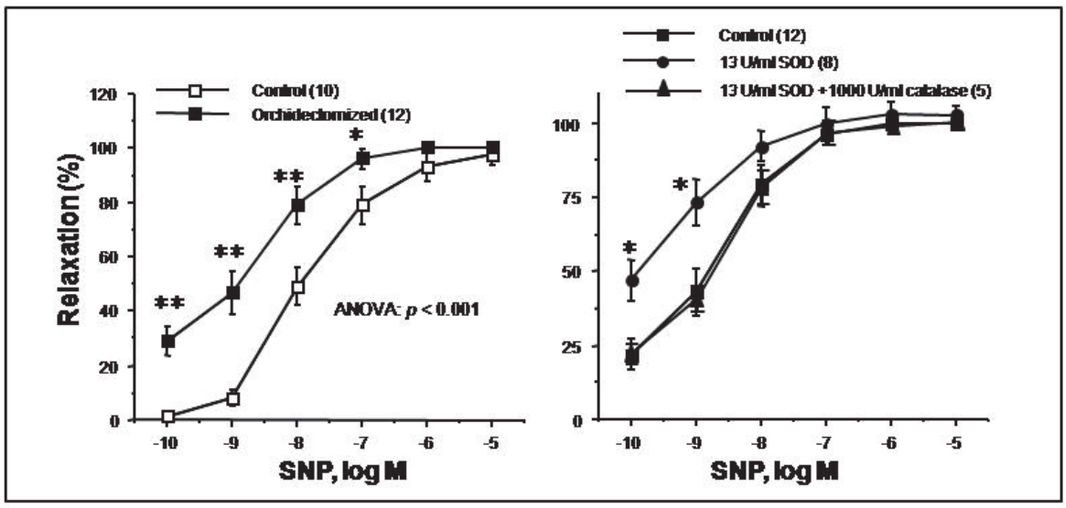

Fig. 5. Effect of orchidectomy, and effect of superoxide dismutase (SOD) and SOD plus catalase on the concentration-response curves to sodium nitroprusside (SNP) in mesenteric artery segments from orchidectomized rats. Results are expressed as a percentage of the inhibition of contraction induced by noradrenaline. Number of animals is indicated in parentheses. *p<0.01 compared with control (from Martín et al., 2005).

At this point, it has been discussed that the loss of male sex hormones did not modify:
(1) the nNOS-derived NO release induced by EFS although it did increase the NO metabolism through superoxide anion and peroxynitrite generation; however, the functional role of the nNOS-derived NO release was more pronounced in arteries from orchidectomized animals, due to products generated from the NO metabolism, such as peroxynitrite and hydrogen peroxide, that seem to be able to compensate for the loss of NO bioavailability, probably through their direct vasodilator effect,
(2) the eNOS-derived NO release induced by Ach in both aorta and mesenteric artery; however the functional role of the endothelial NO release seem to be different in both arteries: Ach-induced relaxation is totally dependent of NO because the Ach-induced relaxation is inhibited when the synthesis of NO is blocked (Blanco-Rivero *et al*., 2007), as occur in aorta from control rats, while in aorta from orchidectomized rats important relaxation exist even after NO synthesis was blocked (Ferrer *et al*., 1999).
These differences could be due to the existence of regulatory mechanisms turned on by the loss of gonadal function that include, at least, prostanoids production.

4. Androgens and prostanoids

Endothelial cells also release vasoconstrictor and vasodilator prostanoids, originated from the arachidonic acid metabolism through the cyclooxygenase (COX) pathway, to regulate vascular tone (Blanco-Rivero *et al*., 2005; Feletou & Vanhoutte, 2006; Henrion *et al*., 1997).

Two major isoforms of COX exist: COX-1 is expressed constitutively and is usually abundant in all animal and human endothelial cells, whereas endothelial COX-2 is induced mainly during inflammatory responses in nearly all animals. Depending on the vessel studied and the agonist used to activate cells, different prostanoids can be released and contribute to vasomotor response depending on the prostanoid receptor that result activated. Among all the prostanoids, one of the most studied is thromboxane A_2 (TXA_2), which has been implicated as a mediator in diseases such as myocardial infarction, hypertension, stroke and bronchial asthma (FitzGerald *et al*., 1987; Narumiya *et al*., 1999; Noll& Luscher, 1998). However, little information is available on the effect of androgens on vascular effects of endogenous TXA_2. Thus, orchidectomy has been reported to either decrease (Gonzales *et al*., 2005) or not modify (Blanco-Rivero *et al*., 2006) TXA_2 synthase expression. Likewise, the contractile effect induced by the TXA_2 mimetic, U-46619, was not modified by orchidectomy in mesenteric (Blanco-Rivero *et al*., 2006) and cerebral (Gonzales *et al*., 2005) rat arteries.

On the other hand, the role of COX-derivates, other than TXA_2, such as prostaglandin (PG) I_2, $PGF_{2\alpha}$ and PGE_2, which can act as vasoconstrictors under certain pathological conditions, is the objective of numerous studies (Félétou *et al*., 2010; Gluais *et al*., 2005; Rapoport & Williams, 1996). However, to the best of our knowledge studies analyzing the effect of endogenous male sex hormones in the involvement of these prostanoids in vascular function were lacking, and therefore this was one of our objectives. As above commented, we have previously demonstrated that EFS induced similar contractile response (Fig.1) in mesenteric arteries from control and orchidectomized rats, responses that appear to be mediated by NA release from adrenergic nerve terminals and the subsequent activation of α-adrenoceptors (Martín *et al*., 2005); in addition, we found that the contractile response to exogenous NA was decreased by orchidectomy, suggesting the EFS could increase NA release in arteries from orchidectomized rats; however, we later demonstrated the EFS-induced NA release was not modified by orchidectomy (Blanco-Rivero *et al*., 2006) which indicated that other vasoconstrictor factors could be released when the artery was electrically stimulated. We observed that the EFS induced a greater TXA_2 formation in arteries from orchidectomized than control rats, which was in line with other reports showing increased TXA_2 release after activation of muscarinic (Blanco-Rivero *et al*., 2007) or α_2-adrenoceptors (Blanco-Rivero *et al*., 2006), and it confirmed the endothelial and smooth muscle cells as sources of TXA_2 production. The increased TXA_2 release could be the contractile factor that was released when the artery is electrically stimulated, and explains 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.

It has been previously reported that prostanoids other than TXA_2, i.e. prostaglandin (PG) I_2 increased neuronal NO release (Ferrer *et al*., 2004) and taking into account that in our experimental conditions the main neurotransmitters involved in that response elicited by EFS were NO and NA, we investigated the regulation of the NA and NO release and function by TXA_2. We observed that in arteries from control rats, the TXA_2 synthesis inhibition, with furegrelate, increased the neuronal NO release, which was in line with reports describing an inhibitory effect of TXA_2 on inducible (Yamada *et al*., 2003) and endothelial (Miyamoto *et al*., 2007) NO release. The vasodilator response induced by the NO donor, sodium nitroprusside was also increased by furegrelate. Concerning the effects of TXA_2 on NA release, inhibition (Nishihara *et al*., 2000) or non-modification (Rump &

Schollmeyer, 1989) have been reported. In our experimental model, the inhibition of TXA_2 synthesis did not modify either NA release or its vasomotor effect, which indicated that endogenous TXA_2 did not alter the function of sympathetic innervations on arteries from control rats. By contrast, in arteries from orchidectomized rats, endogenous TXA_2 did not regulate either the release/function of neuronal NO or NA. The results obtained in mesenteric arteries from control animals explain the decreased EFS-induced response in the presence of furegrelate; however, did not explain the unaltered EFS-induced response in arteries from orchidectomized rats, since to remove a constrictor substance a decreased in EFS-induced vasoconstriction would be expected. Since cross-talk between TXA_2 and PGI_2 has been reported (Cheng *et al*., 2002; Martorell *et al*., 2008) and joint increases in PGI_2 and TXA_2 synthesis have been shown in pathological conditions (Caughey *et al*., 2001; FitzGerald, 1991), the effect of TXA_2 on PGI_2 production was analyzed, as well as, its dependence of the hormonal status. We observed that the inhibition of endogenous synthesis of TXA_2 did not modify the release of PGI_2 in arteries from control rats, while it did greatly increase PGI_2 release in arteries from orchidectomized rats, which could work as a contractile factor after activation of TXA_2 receptors. Taking together these results, the loss of gonadal function in male rats increased the non-endothelial TXA_2 release in mesenteric arteries, and regulates the EFS-induced response through different mechanisms.

As previously commented, orchidectomy increased the TXA_2 release in endothelial-intact mesenteric arteries (Blanco-Rivero *et al*., 2006; 2007). Similar effect was also observed in aorta segments from comparable animals (Martorell *et al*., 2008), however, in this case the relaxation to ACh was increased. Based on this result, the level of expression of COX-2 as well as the production of prostanoids derived from COX-2 other than TXA_2 was investigated. We found that COX-2 expression, in contrast to observations in mesenteric artery (Blanco-Rivero *et al*., 2006), was increased in aortas from orchidectomized rats indicating that endogenous male sex hormones act differently depending on the specific vessel. Our results also show that, in aorta from orchidectomized rats, COX-2 derivatives could also be increased and play a role in the regulation of vascular function. To test this hypothesis, we analyzed the effect of the COX-2 inhibitor NS-398 on the ACh-induced response. In contrast to our assumptions, we found that NS-398 did not modify the ACh-induced relaxation in either group of rats, apparently indicating the lack of participation of COX-2 derived products in the ACh response, probably due to the equilibrium between the inhibition of prostanoids formation and phosphodiesterase inhibition (Klein *et al*., 2007), which allowed us to speculate that the contribution of different prostanoids to the vasodilator response mediated by ACh could be regulated by endogenous male sex hormones.

Once we had established that orchidectomy increased TXA_2 release, we analyzed the possible role of this prostanoid in the response to ACh by analyzing the effect of the TXA2 synthase inhibitor, furegrelate, and the TP receptor antagonist, SQ29,548, on the vasodilator response to ACh. We observed that neither substance had any effect on the ACh-induced response in arteries from control animals, indicating that TXA_2 did not participate in that response, in agreement with reports in other rat strains (Gluais *et al*., 2005; Rapoport & Williams, 1996). However, in arteries from orchidectomized rats, furegrelate enhanced the vasodilator response to ACh, showing a functional involvement of TXA_2. The fact that the contractile response to the TXA_2 mimetic U-46619 was similar in arteries from control and orchidectomized rats demonstrated that sensitivity to TXA2 is not modified by orchidectomy, which agrees with reports in cerebral (Gonzales *et al*., 2005) and mesenteric

(Blanco-Rivero *et al*., 2006) arteries; additionally, it also shows that differences in the TXA_2 involvement in the ACh-response are due to increased synthesis rather than increased sensitivity to TXA_2. However, the incubation with SQ29,548 did not affect the ACh-induced relaxation, which seems to contradict the results obtained with furegrelate. However, since interactions among different prostanoids have been reported (Bachschmid *et al*., 2005; Cheng *et al*., 2002), it is possible to hypothesize that when TXA_2 synthesis is inhibited, the production of other prostanoids, which counterbalance the TXA_2 effect, could be increased. Therefore, we investigated the effect of inhibiting PGI_2 synthesis (with TCP) on the ACh-induced response, observing that it decreased the vasodilator response to ACh to a greater extent in arteries from orchidectomized than in those of control rats, which would indicate a greater involvement of this vasodilator prostanoid in the former arteries. Therefore, the release and vasomotor effect of PGI_2 was investigated. The ACh-induced PGI_2 release was increased in arteries from orchidectomized rats, probably due to the superoxide anion overproduction observed in aortas from orchidectomized rats (Blanco-Rivero *et al*., 2006), supporting the concept of redox regulation of vascular prostanoid synthesis proposed by Bachschmid *et al*. (2005). Moreover, the increased production of PGI_2 is in line with that reported in human syndromes involving platelet activation in which PGI_2 biosynthesis is elevated along with TXA_2 (Caughey *et al*., 2001; FitzGerald, 1991). It is known that PGI_2 can induce both vasodilation, through activation of prostacyclin receptors (IP) and thereby increasing cyclic-AMP, and vasoconstriction through activation of TP receptors (Blanco-Rivero *et al*., 2005). In the present study, we found that exogenous PGI_2 induced relaxation in rat aorta, and that it was decreased in arteries from orchidectomized rats, which could be due to differences in the expression of IP receptors rather than differences in cell signalling operating after receptor activation; we have observed that the relaxation induced by the activator of adenylate cyclase, forskolin, was similar in arteries from control and orchidectomized rats (unpublished data).

Since considerable evidence exists for cross-talk between the TXA_2 and PGI_2 systems (Cheng *et al*., 2002), we analyzed the functional effect of inhibiting the synthesis of both prostanoids. We observed that co-incubation of arteries with TCP plus furegrelate, or TCP plus SQ29,548, reversed the decreased response to ACh caused by TCP in arteries from control rats, showing the existence of a balance between TXA_2 and PGI_2 in these arteries. However, in arteries from orchidectomized rats, the co-incubation with TCP plus furegrelate did not modify the decreased ACh response caused by TCP, indicating the participation of prostanoids other than PGI_2 and TXA_2 that could induce contraction. Moreover, these prostanoids would activate TP receptors since co-incubation with TCP and SQ29,548 completely reversed the decrease in the ACh response induced by TCP.

Among COX-2 derivatives, other than TXA_2 and PGI_2, that can activate TP receptors, PGE_2 is the most plausible candidate (Gluais *et al*., 2005; Blanco-Rivero *et al*., 2007) since the ACh-induced $PGF_{2\alpha}$ production and its vasoconstrictor effect were both very limited. Therefore, we investigated the ACh-induced PGE_2 release, as well as its vasoconstrictor effect. We found that both ACh-induced PGE_2 production and PGE_2-induced vasoconstrictor response were greater in arteries from orchidectomized than in those of control rats. Consequently, the ACh-induced PGE_2 release, under inhibited synthesis of TXA_2 and PGI_2, was analyzed. We found the ACh-induced PGE_2 production further increased, probably as a consequence of increased PGH_2 production and subsequent transformation into PGE_2 (Frein *et al*., 2005); and, what is more important, the PGE_2 increase was more pronounced in arteries from orchidectomized than in those of control rats.

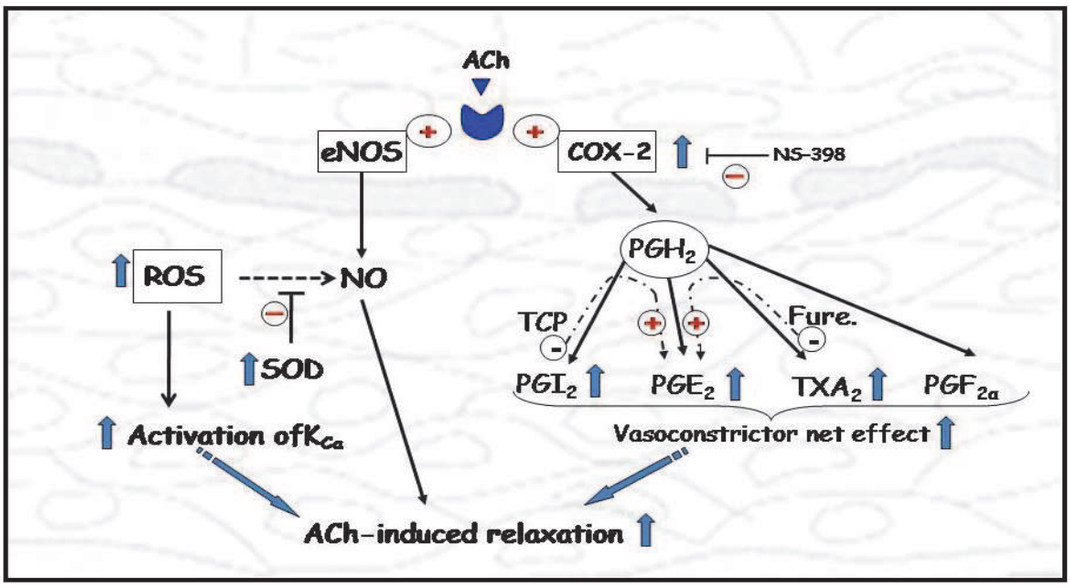

Fig. 6. Scheme showing the effect of orchidectomy on different mechanisms involved in the ACh-induced relaxation (see text for explanation).

This finding confirms our hypothesis that when the synthesis of PGI_2 and TXA_2 was inhibited, the release of PGE_2 was increased in arteries from orchidectomized rats, but raises the question as to why the PGE_2 produced in the presence of TCP plus furegrelate did not affect the ACh-induced relaxation in arteries from control animals. The possible explanation could be that the PGE_2 release was not sufficient to induce a vasomotor effect and/or the PGE_2-induced contraction in arteries from control rats was diminished as a consequence of different expression of EP receptor subtypes. By itself, this finding is of physiological relevance, since PGE_2 release and the vasoconstrictor effect are both increased in orchidectomized animals. In summary, in rat aorta orchidectomy enhances COX-2 expression, and induces an imbalance in the production and function of vasodilator and vasoconstrictor prostanoids, in such a way that the vasoconstrictor prostanoids predominate in the latter group. Additionally, we have previously reported a decreased NO bioavailability in aorta from orchidectomized rats (Blanco-Rivero *et al*., 2006) that would also counteract the vasodilator response to ACh. However, despite these findings, the vasodilator response to ACh is increased in aorta from orchidectomized rats, probably as a consequence of compensatory mechanisms, such as the activation of K_{Ca} channels by superoxide anion, the formation of which is increased in orchidectomized rats (Ferrer *et al*., 1999) (Fig. 6).

5. Androgens and NO release, oxidative stress and protein kinase C interactions

The results described above point to that orchidectomy increases the oxidative stress through superoxide anion formation, but in contrast to that expected, without modifying the NO release. What is the mechanism underlying this effect?.

In several types of vascular diseases, protein kinase C (PKC) activation is involved in the induction of oxidative stress through the increased expression and activity of NADPH oxidase and the eNOS uncoupling (Hadi & Swaidi, 2007; Vanhoutte, 2001). But increased

of PKC activity by different reactive oxygen species has been also reported (Balafanova *et al*., 2002; Bapat *et al*., 2001; Oeckler & Wolin, 2000). PKC is a ubiquitous enzyme that was originally described as calcium-activated, phospholipid-dependent protein kinase. Molecular cloning and biochemical analysis have revealed a family of PKC subspecies with closely related structures (Newton, 1995; Nishizuka, 1992). The activity of these proteins is critical for signal transduction of a wide range of biological responses (Chen, 2003; Nishizuka, 1984). The pathways through PKC exerts its vascular effects include actions on ion channels, cytoskeleton and cell adhesion proteins, transcription factors, other kinases and other proteins (Spitaler & Cantrell, 2004; Ward *et al*., 2004). The participation of PKC in vascular smooth muscle contraction is well established (Khalil & van Breemen, 1988; Salamanca & Khalil, 2005); in addition, different investigations have also reveal that PKC can phosphorylate nNOS in the central nervous system leading to an increase (Nakane *et al*., 1991; Okada, 1995) or a decrease (Bredt *et al*., 1992; Dawson *et al*., 1993) in neuronal NO production.

On the other hand, influence of gender on PKC activity and expression had been studied (Kanashiro & Khalil, 2001). In that work, the authors observed a gender-specific reduction in vascular smooth muscle reactivity in female rats with intact gonadal function compared with males, that was associated with a reduction in the expression and activity of different α-, δ-, and ζ-PKC isoforms; they also proposed that gender-specific differences in vascular reactivity and PKC activity were possible related to endogenous estrogen. However, little information is available on the specific effect of endogenous male sex hormones on PKC activity in vascular tissues.

As described in the section corresponding to neuronal NO release, we observed that the neuronal NO release was not modified by orchidectomy although the nNOS expression was diminished, which indicated that nNOS activity could be increased; regarding regulation of nNOS by male sex hormones both increase (Simoncini *et al*., 2003; Weiner *et al*., 1994) or decrease (Reynoso *et al*., 2002; Singh *et al*., 2000) have been reported. Since in our experimental model, orchidectomy induced an increased oxidative stress, we analyzed the possible differences in PKC activity as well as its involvement in neuronal NO release.

We observed that orchidectomy increased PKC activity in rat mesenteric arteries (Blanco-Rivero *et al*., 2005), in contrast to the results obtained in aorta from Wistar-Kyoto rats (Kanashiro & Khalil, 2001); this discrepancy could be due to differences in the artery analyzed, since it has been described different enzyme properties and function in the same blood vessels from different species and in different vessels from the same species (Liou & Morgan, 1994; Kanashiro & Khalil, 1988;Khalil *etal*., 1992). However, the fact that PKC was increased by orchidectomy is not totally surprising, since orchidectomy increased the formation of superoxide anion and peroxynitrite that has been described to act in cell signalling pathways, for instance, increased PKC activity (Balafanova *et al*., 2002; Bapat *et al*., 2001; Oeckler & Wolin, 2000).

The regulatory effect of PKC on nNOS activity in arteries from the orchidectomized and the control animals was analyzed by using DAF-2 as fluorescence probe to measure the modification in basal and EFS-induced NO release. The results showed that the PKC activator PDBu (Abdel-Latif, 1986; Nishizuka, 1984) induced a greater increase in EFS-induced NO release in arteries from control than from orchidectomized animals, while the PKC inhibitor, calphostin C (Kobayashi *et al*., 1989), induced a stronger decrease in arteries from the control than the orchidectomized animals (Fig. 7).

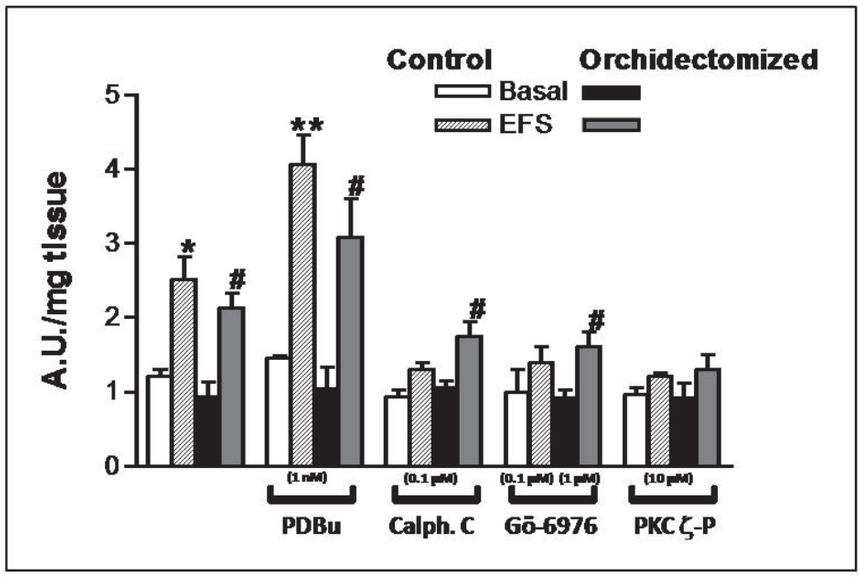

Fig. 7. Effect of PDBu, calphostin, Gö-6976 or PKCζ-PI on the basal-and EFS-induced NO release in mesenteric arteries from control and orchidectomized rats. Results are expressed as arbitrary units (AU). Number of animals=4-7. *p<0.05, **p<0.01 *vs* the respective basal NO release from control rats; #p<0.05 *vs* the respective basal NO release from orchidectomized rats (from Blanco-Rivero et al., 2005).

These results indicate that PKC is involved in nNOS activation in arteries from both control and orchidectomized rats. Because the degree of PKC activation is already greater in arteries from orchidectomized rats, PDBu and calphostin C showed less ability to respectively increase or diminish NO release in arteries from orchidectomized than from control rats. Conventional and novel PKC isoforms are dependent on dyacylglycerol (DAG) (Ward *et al*., 2004) and, since we use the pharmacological mimetic of DAG, PDBu, we stimulate both isoforms. Although selective inhibitors for these isoforms do not exist (Davies *et al*., 2000) it has been reported that certain compounds, such as the indolocarbazole Gö6976, are partially selective for conventional over the novel and atypical isoforms (Martiny-Baron *et al*., 1993; Ward *et al*., 2004). Consequently, we tested the effect of this inhibitor on nNOS activity, and observed that a concentration of 0.1 μM Gö6976 inhibited the EFS-induced neuronal NO release in arteries from control arteries while it did not affect the neuronal NO release in arteries from orchidectomized rats. A higher Gö6976 concentration (1 μM) was used but it only decreased the EFS-induced NO release in arteries from orchidectomized rats. All these results support the assumption that nNOS seems to be much more activated by PKC, and probably the conventional PKC isoforms, in arteries from the orchidectomized than from the control rats.

Since the atypical PKC ζ isoform has been described in vascular smooth muscle as modulating vascular responses to different agents (Cogolludo *et al*., 2003; De Witt *et al*., 2001), the effect of this isoform on nNOS activity was analyzed. We found that PKCζ-PI decreased EFS-induced NO release to a similar extent in arteries from control and orchidectomized animals. This result indicates that PKC ζ isoform involvement in the regulation of neuronal NO release occurs and that this involvement is not modulated by endogenous male sex hormones.

Regarding endothelial NO release, it has been previously described that orchidectomy did not modify the Ach-stimulated endothelial NO release, despite the increased production of superoxide anion. Based on the results obtained about PKC regulation of nNOS activity, the action of PKC activation or inhibition on eNOS activity was also investigated in arteries from orchidectomized and control animals. The results obtained showed that neither the PKC activator, PDBu (Nishizuka, 1984), nor the PKC inhibitor, calphostin C (Kobayashi *et al*. 1989), modified ACh-induced NO release in arteries from control animals. In contrast, in arteries from orchidectomized rats, PKC activation increased the basal and the ACh-induced NO release while PKC inhibition more strongly decreased both basal and ACh-induced NO release (Fig. 8).

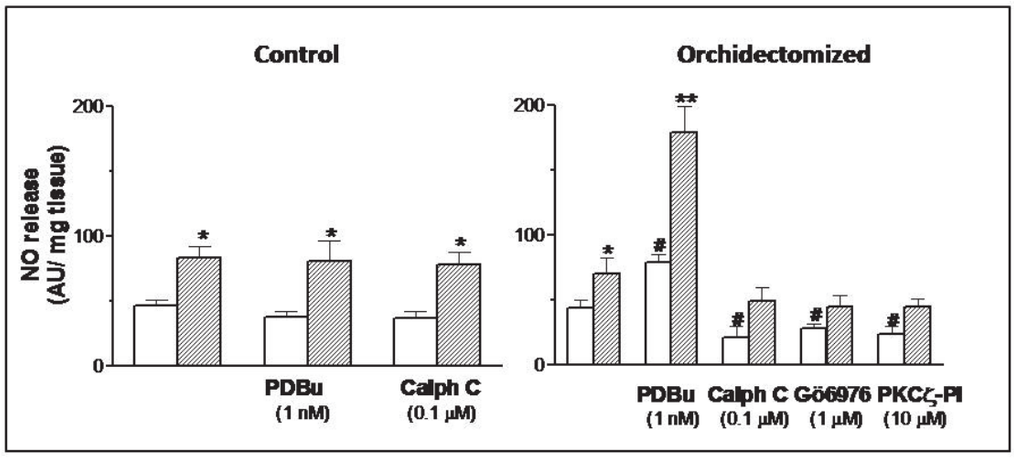

Fig. 8. Effect of PDBu, calphostin, Gö-6976 or PKCζ-PI on the basal - (white columns) and Ach - (dashed columns) induced NO release in mesenteric arteries from control and orchidectomized rats. Results are expressed as arbitrary units (AU). Number of animals=8. **p*<0.05 *vs* the respective basal NO release; #*p*<0.05 *vs* basal NO release in non treated arteries (from Blanco-Rivero et al., 2007).

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 contrast with the results reported with nNOS (Blanco-Rivero *et al*., 2005) in which we observed an 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. In this regard, it is important to keep in mind that regulatory mechanisms other than PKC could be working on eNOS, including different redox conditions (Polytarchou & Papadimitriou, 2005), phosphatases activity (Fleming *et al*., 2001) and/or other kinases (Ferrer *et al*., 2004) that, in their turn, regulate the intracellular environment and function. Additionally, since the pharmacological mimic of dyacylglycerol, PDBu, stimulate 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 PKCζ 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*., 2005), we also tested the possible involvement of this isoform in endothelial NO release. The fact that PKCζ-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 PKCζ-PI, diminished both basal and ACh-induced NO release, it seems that eNOS, like nNOS (Blanco-Rivero *et al*., 2005), would have already been activated by PKC in arteries from orchidectomized rats.

These results show that PKC activity is enhanced in mesenteric arteries from orchidectomized rats, and this increase would be responsible for the higher nNOS and eNOS activity.

6. Conclusions

Orchidectomy alters different cell signalling pathways that are involved in vascular tone regulation. Orchidectomy increases: (i) the formation of superoxide anion and peroxynitrite; (ii) the activity of PKC; and (iii) the expression of COX-2, the production of prostanoids derived from COX-2, as well as their vasoconstrictor effect. These aspects seem to be physiologically relevant, since the balance between vasodilator/vasoconstrictor prostanoids is lost in favour of vasoconstrictor substances in arteries from orchidectomized rats. This situation could indicate a disadvantage in cardiovascular function in the absence of male sex hormones, thereby suggesting that testosterone has a beneficial influence on the vasculature. However, in the animals used in our study (6 months old) several compensatory mechanisms are working: reactive oxygen species are able to induce relaxation; PKC positively regulates nNOS and eNOS activity ensuring the maintenance of NO release; the activity and expression of SOD are increased in an attempt to compensate for the increased superoxide anion production. This intriguing information makes it essential to perform studies in vascular function taking into account different cell signalling pathways that are working simultaneously. Future studies in the research field of androgens on cell signaling pathways are needed, since they will be of important interest to implement therapeutic strategies that could improve vascular function.

7. Acknowledgements

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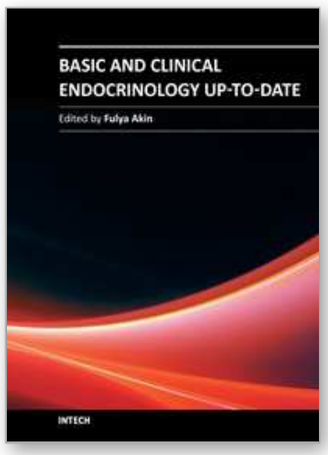

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This book provides the most up-to-date information on the basic and clinical aspects of endocrinology. It offers both researchers and clinicians experts, gold-standard analysis of endocrine research and translation into the treatment of diseases such as insulinoma, endocrine disease in pregnancy and steroid induced osteoporosis. Investigates both the endocrine functions of the kidneys and how the kidney acts as a target for hormones from other organ systems. Presents a uniquely comprehensive look at all aspects of endocrine changes in pregnancy and cardiovascular effects of androgens.

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