Available online at www.sciencedirect.com

ScienceDirect

European Journal of Pharmacology 578 (2008) 261–269

www.elsevier.com/locate/ejphar

Negative inotropic effect of selective AT_2 receptor stimulation and its modulation by the endocardial endothelium

Paulo Castro-Chaves [1], Susana Soares [1], Ricardo Fontes-Carvalho, Adelino F. Leite-Moreira *

Department of Physiology, Faculty of Medicine, Alameda Prof. Hernâni Monteiro, 4200-319 Porto, Portugal

Received 25 October 2006; received in revised form 17 September 2007; accepted 18 September 2007
Available online 2 October 2007

Abstract

Angiotensin II is an octapeptide whose effects are mediated by two types of receptors. AT_1 receptors are responsible for the vasoconstrictor, positive inotropic and growth promoting properties, while AT_2 receptors have been linked to vasodilator and anti-mitogenic properties. In this study we investigated the effects of selective AT_2 receptor stimulation on myocardial contractility and lusitropy. Effects of selective AT_2 receptor activation were evaluated in rabbit right papillary muscles ($n=96$) by adding increasing concentrations of H-9395, an AT_2 receptor agonist, alone or in presence of a selective AT_1 receptor antagonist (ZD-7155), or alternatively, by adding increasing concentrations of angiotensin II in presence of ZD-7155. In the latter conditions, selective AT_2 receptor activation was also performed in presence of NG-nitro-L-Arginine, indomethacin, proadifen, hydroxocobalamin, apamin plus charybdotoxin, Hoe-140 or PD-123,319, as well as, after endocardial endothelium removal. Selective AT_2 stimulation induced a negative inotropic and lusitropic effect in the first three protocols. This effect was completely abolished after selective removal of the endocardial endothelium and blunted in presence of Hoe-140, hydroxocobalamin, apamin plus charybdotoxin and PD-123,319, but maintained in presence of NG-nitro-L-Arginine, indomethacin or proadifen. Selective AT_2 receptor stimulation induces a negative inotropic and lusitropic effect, which is modulated by endocardial endothelium and mediated by bradykinin B_2 receptors through NO release and calcium dependent potassium channels activation. Such findings may help to better understand the therapeutic effects of selective AT_1 antagonists, which are increasingly used for treating cardiovascular diseases.

Keywords: Angiotensin II; AT_2 receptor; Endocardial endothelium; Inotropism

1. Introduction

Angiotensin II mediates its biological actions by binding to distinct membrane-bound receptors and activating multiple intracellular pathways. In 1989, different research teams independently provided evidence for the existence of two major subtypes of angiotensin II receptors (Whitebread et al., 1989; Chiu et al., 1989). These two major subtypes have been identified, cloned and named as AT_1 and AT_2 (de Gasparo et al., 2000). Angiotensin AT_1 receptors are responsible for mediating the classic stimulatory actions of angiotensin II on blood pressure, water and sodium intake, renal sodium retention, secretion of vasopressin and aldosterone and cell growth and proliferation (de Gasparo et al., 2000; Gallinat et al., 2000).

Although the exact physiological functions of angiotensin AT_2 receptors are not established, it is known that these receptors may offset or oppose the angiotensin AT_1 receptor mediated actions of angiotensin II on cell growth, blood pressure and fluid intake (Gallinat et al., 2000). However, the different expression and actions of angiotensin AT_2 receptors within cardiovascular tissues under certain physiological/pathological conditions suggest that there is a far from complete understanding of the role of angiotensin AT_1/AT_2 receptors in cardiovascular regulation (Horiuchi et al., 1999). Angiotensin AT_2 receptor

* Corresponding author. Tel.: +351 22 551 36 44; fax: +351 22 551 36 46.
E-mail address: amoreira@med.up.pt (A.F. Leite-Moreira).
[1] Both authors equally contributed to the work presented in this article.

0014-2999/$ - see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.ejphar.2007.09.026

function is likely to be context-specific, as suggested by Scheider and Lorell (Schneider and Lorell, 2001).

In fetal tissues, angiotensin AT_2 receptor is the predominant subtype expressed. After birth, its expression in mesenchimal tissues rapidly decreases. The common misconception that angiotensin AT_2 receptors do not exist in appreciable amounts in adult animal vasculature is however slowly changing. They are located in many different vessel types, albeit at lower, but functional levels. For example, in rat aorta these receptors constitute about 30–40% of angiotensin II receptors (Chang and Lotti, 1991). In adult rat cardiomyocytes, angiotensin AT_2 receptors are expressed at low levels, being expressed in 8–13% of cardiomyocytes (Busche et al., 2000), but are significantly increased in hypertrophy (36%) and heart failure (112%) when compared with controls (Lopez et al., 1994; Ohkubo et al., 1997; Bartunek et al., 1999). In rabbit hearts, this receptor subtype is almost totally absent in nervous, conductive and atrial tissue but, in the ventricle, approximately 20% of the angiotensin II receptors are AT_2 (Brink et al., 1995). Angiotensin AT_2 receptors also gain particular prominence in adult human heart. In both normal noninfarcted or hypertrophied human hearts, there is a predominance of angiotensin AT_2 receptor binding sites in the myocardium, constituting about 69±4% of angiotensin II binding sites (Regitz-Zagrosek et al., 1995; Matsubara, 1998; de Gasparo et al., 2000; Johren et al., 2004).

There is an increasing amount of literature demonstrating the role of angiotensin AT_2 receptors in the regulation of both acute and chronic cardiovascular function. Chronically, angiotensin AT_2 receptors have been implicated in the inhibition of myocardial hypertrophy (Booz and Baker, 1996; Bartunek et al., 1999), fibroblast proliferation (Tsutsumi et al., 1998) and vascular cell hyperplasia (Nakajima et al., 1995). The overexpression of angiotensin AT_2 receptors in cardiomyocytes attenuates perivascular fibrosis by a kinin/nitric oxide dependent mechanism (Kurisu et al., 2003). In a rat model of chronic heart failure, left ventricular remodeling and cardiac function were improved by blockade of angiotensin AT_1 receptors (Liu et al., 1997). This effect may be partially explained by a higher activation of angiotensin AT_2 receptors.

In the vasculature, it has been demonstrated that acute angiotensin AT_2 receptor stimulation leads to relaxation of isolated arteries, including rabbit renal arterioles (Arima et al., 1997; Endo et al., 1997), rabbit cerebral arteries (Haberl, 1994) and rat mesenteric arteries (Widdop et al., 2002, 2003). In the human heart, angiotensin II exerts an acute vasodilating effect in the coronary microcirculation through angiotensin AT_2 receptor stimulation (Batenburg et al., 2004a,b). However, the effects of acute angiotensin AT_2 stimulation in myocardial function, namely in myocardial inotropy and lusitropy, are not yet known. The main objectives of the present study are to describe the myocardial effects caused by angiotensin AT_2 stimulation and their underlying mechanisms.

2. Materials and methods

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by US National Institutes of Health (NIH Publication No 85-23, revised 1996).

2.1. Experimental preparation

The effects of angiotensin II were studied in isolated right papillary muscles of New Zealand White rabbits (Oryctolagus cuniculus; 2.7±0.12 kg). Rabbits were anaesthetized with sodium pentobarbital (25 mg/kg, iv) and the heart was quickly excised. The tissues were immersed in a modified Krebs–Ringer solution, at 35°C, with cardioplegic 2,3-butanedione monoxime (3%) and calf serum (5%; Bio-Whittaker, St. Louis, Maryland, USA). The modified Krebs–Ringer solutions contained (in mM): NaCl 98, KCl 4.7, $MgSO_4$ 2.4, KH_2PO_4 1.2, $CaCl_2$ 1.8, $NaHCO_3$ 20, CH_3COONa 5, $C_3H_3O_3Na$ 15, glucose 4.5 and atenolol 0.02. Atenolol was used to prevent β-adrenergic mediated effects. The solutions were in equilibrium with 95% O_2 and 5% CO_2, maintaining the pH between 7.38-7.42. Rabbit papillary muscles (n=96; length: 3.2±1.1 mm; weight: 2.2±1.3 mg; cross-sectional area: 0.6±0.3 mm2; preload: 5.0±1.1 mN) were then carefully dissected. Afterwards, they were vertically mounted in a 10 ml plexi glass organ bath. The lower muscular end was fixed in a phosphorbronze clip and the upper tendinous end was attached to an electromagnetic length-tension transducer (University of Antwerp, Belgium). Preload was initially set between 3 and 4 mN according to muscle dimensions. The preparations were stimulated at 0.6 Hz with a voltage of 10% above threshold (typically 3–6 mV) by rectangular pulses of 5 ms duration through two platinum electrodes arranged longitudinally alongside the entire muscle. Twenty minutes later, bathing solutions were replaced by corresponding Krebs–Ringer solutions without 2,3-butanedione monoxime. During the next 2 h, muscles were stabilized. Bathing solutions were then replaced by corresponding Krebs–Ringer solutions without calf serum and L_{max} was calculated. Protocols were initiated after obtaining two similar isotonic and isometric control twitches separated by a 10 min in interval.

2.2. Experimental protocols

In a first set of protocols, we studied the effects of selective angiotensin AT_2 receptor stimulation. H-9395 (Nicotinoyl-Tyr-Lys(Z-Arg)-His-Pro-Ile-OH, supplied from Bachem AG), an agonist of angiotensin AT_2 receptors, was added to the superfusing solution in increasing concentrations (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) in basal conditions (n=15) and in the presence of a selective antagonist of angiotensin AT_1 receptors (ZD-7155; 10^{-7} M; n=9). We also tested the effects of the addition to the superfusing solution of increasing concentrations of angiotensin II (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) in presence of a selective angiotensin AT_1 receptor antagonist (ZD7155, 10^{-6} M; n=12). ZD-7155 is an angiotensin AT_1 receptor competitive antagonist that is approximately ten times more potent than losartan in suppressing the angiotensin II-induced pressor response (Junggren et al., 1996).

In a second set of protocols, we studied the underlying mechanisms to the inotropic effect observed after selective angiotensin AT_2 receptor stimulation. This was performed by adding increasing concentrations of angiotensin II (10^{-8}, 10^{-7},

10^{-6}, 10^{-5} M) in presence of ZD-7155 (5,7-Diethyl-3,4-dihydro-1-[[2′-(1H-tetrazol-5-yl)[1,1′-biphenyl]-4-yl]methyl]-1,6-naphthyridin-2(1H)-onehydrochloride; 10^{-6} M) in different experimental conditions: after removal of the endocardial endothelium ($n=7$) and in the presence of NG-nitro-L-Arginine ($3\ 10^{-5}$ M; $n=9$), indomethacin (10^{-5} M; $n=7$), proadifen (10^{-6} M; $n=9$), Hoe-140 (D-Arg-L-Arg-L-Pro-L-Hyp-Gly-L-(2-thienyl)Ala-L-Ser-D-1,2,3,4-tetrahydro-3-isoquinolinecarbonyl-L-(2α,3β,7aβ)-octahydro-1H-indole-2-carbonyl-L-Arg; 10^{-7} M; $n=6$), hydroxocobalamin (10^{-4} M; $n=7$) and charybdotoxin plus apamin (10^{-7} M; $n=9$). These later substances are inhibitors of the synthesis of nitric oxide, prostaglandins or endothelium-derived hyperpolarizing factor (through the inhibition of cytochrome P-450 monooxygenase enzymes), an antagonist of bradykinin B_2 receptors, a nitric oxide scavenger, an IK_{Ca} and BK_{Ca} channel inhibitor and a SK_{Ca} channel inhibitor, respectively.

The concentrations of NG-nitro-L-arginine, indomethacin, hydroxocobalamin, apamin, charybdotoxin and proadifen were selected on the basis of several studies showing that their physiological effects in myocardial tissue preparations or whole heart preparations are exerted by concentrations in the micromolar range (Mohan et al., 1995; Kato et al., 2001; Batenburg et al., 2004a,b; Berges et al., 2005).

Finally, the effects of angiotensin II in presence of ZD-7155 were evaluated after selective inhibition of angiotensin AT_2 receptors with PD-123,319 (S-(+)-1-[(4-(Dimethylamino)-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid di(trifluoroacetate) salt hydrate; 10^{-6} M; $n=5$).

Selective removal of the endocardial endothelium was performed according to the methodology described by Brutsaert and collaborators (Brutsaert et al., 1988). Briefly, this consisted in the immersion of the papillary muscles in a 0.5% solution of Triton X-100 during 1 s, followed by an abundant washout. Of note, that in each experimental protocol all papillary muscles were obtained from different animals. All chemicals, except H-9395, were purchased from Sigma Chemical Co, St Louis, Mo.

2.3. Data analysis

Isotonic and isometric twitches were recorded and analyzed with dedicated software (University of Antwerp, Belgium). Selected parameters include: active tension (mN mm^{-2}); maximum velocity of tension rise (dT/dt_{max}, mN mm^{-2} s^{-1}); maximum velocity of tension decline (dT/dt_{min}, mN mm^{-2} s^{-1}); peak isotonic shortening (%L_{max}); maximum velocity of shortening (dL/dt_{max}, L_{max} s^{-1}); maximum velocity of lengthening (dL/dt_{min}, L_{max} s^{-1}), time to half relaxation (ms) and resting tension (mN mm^{-2}).

2.4. Statistical methods

Values are means ± S.E.M. Statistical significance was determined using analysis of variances (ANOVA) and Student–Newman–Keuls for pairwise multiple comparisons. $P<0.05$ was accepted as significant.

3. Results

Baseline performance of rabbit papillary muscles was similar in all experimental protocols. Mean values of the papillary muscles contractile parameters were: active tension 27.9 ± 2.5 mN/mm^2; dT/dt_{max} 185 ± 15 mN/mm^2 s; dT/dt_{min} −161 ± 13 mN/mm^2 s; peak shortening 10 ± 0.8% of L_{max}; dL/dt_{max} 0.72 ± 0.04 $L_{max}\cdot s^{-1}$; dL/dt_{min} −2.4 ± 0.17 $L_{max}\cdot s^{-1}$; time to half relaxation 385 ± 10 ms.

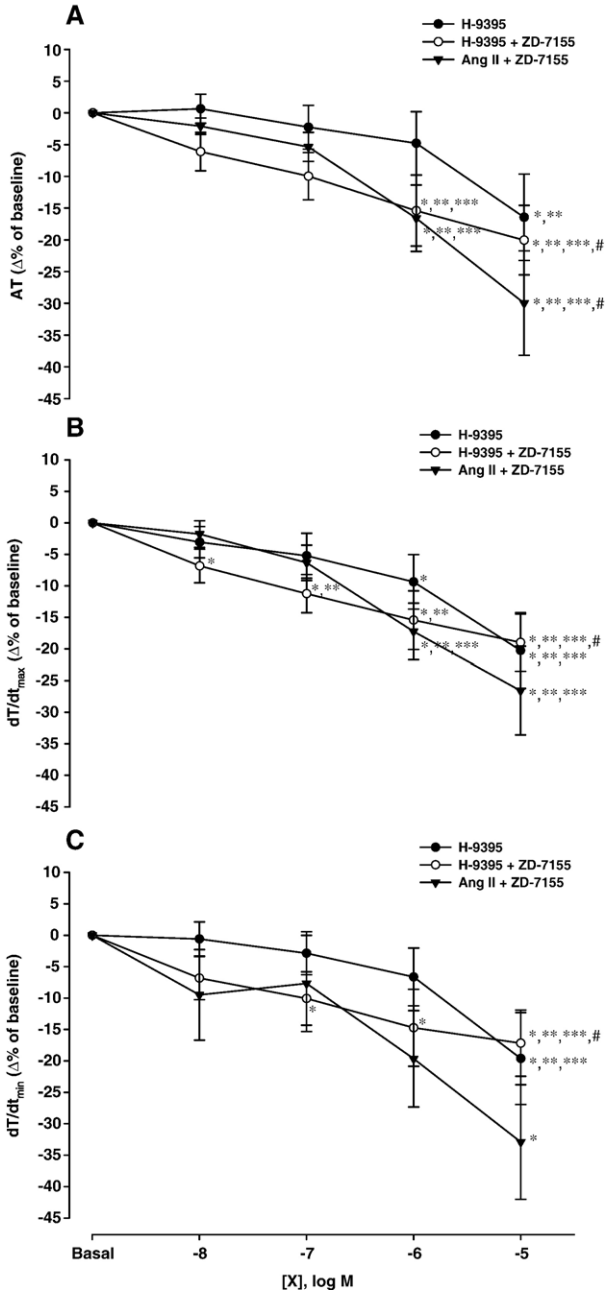

Fig. 1. Effects of increasing concentrations of H-9395, H-9395 plus ZD-7155 and angiotensin II plus ZD-7155 on active tension (AT; A) and peak rate of tension rise (dT/dt_{max}; B) or tension decline (dT/dt_{min}; C). On the axis, [*X*] represents the concentrations of angiotensin II or H-9395, according to the respective protocol. Selective stimulation of AT_2 receptors induced negative inotropic and lusitropic effects maximal for the concentration of 10^{-5} M. *$P<0.05$ vs. basal; **$P<0.05$ vs. 10^{-8} M; ***$P<0.05$ vs. 10^{-7} M; #$P<0.05$ vs. 10^{-6} M.

Selective stimulation of angiotensin AT_2 receptors with the agonist H-9395 induced a concentration-dependent negative inotropic effect (Fig. 1). This effect was maximal for the concentration of 10^{-5} M, decreasing 16.4±6.8% active tension, 20.3±6.0% dT/dt_{max}, 19.6±7.3% dT/dt_{min}, 16.0±5.6% peak shortening and 32.3±12.2% dL/dt_{max} ($P<0.05$). The addition of the same agonist in the presence of a selective angiotensin AT_1 receptor antagonist (ZD-7155) increased the selectivity of the stimulation and caused a slight amplification of the negative inotropic effect (Fig. 1). Selective angiotensin AT_2 stimulation using increasing concentrations of angiotensin II in the presence of ZD-7155 also induced a dose-dependent negative inotropic effect, decreasing at the maximal concentration of angiotensin II 29.9±8.2% active tension, 26.6±7.0% dT/dt_{max}, 32.9±9.1% dT/dt_{min}, 26.8±7.0% peak shortening and 22.4±5.8% dL/dt_{max}. This effect was not significantly different from the one observed with the addition of H-9395 in the presence of ZD-7155.

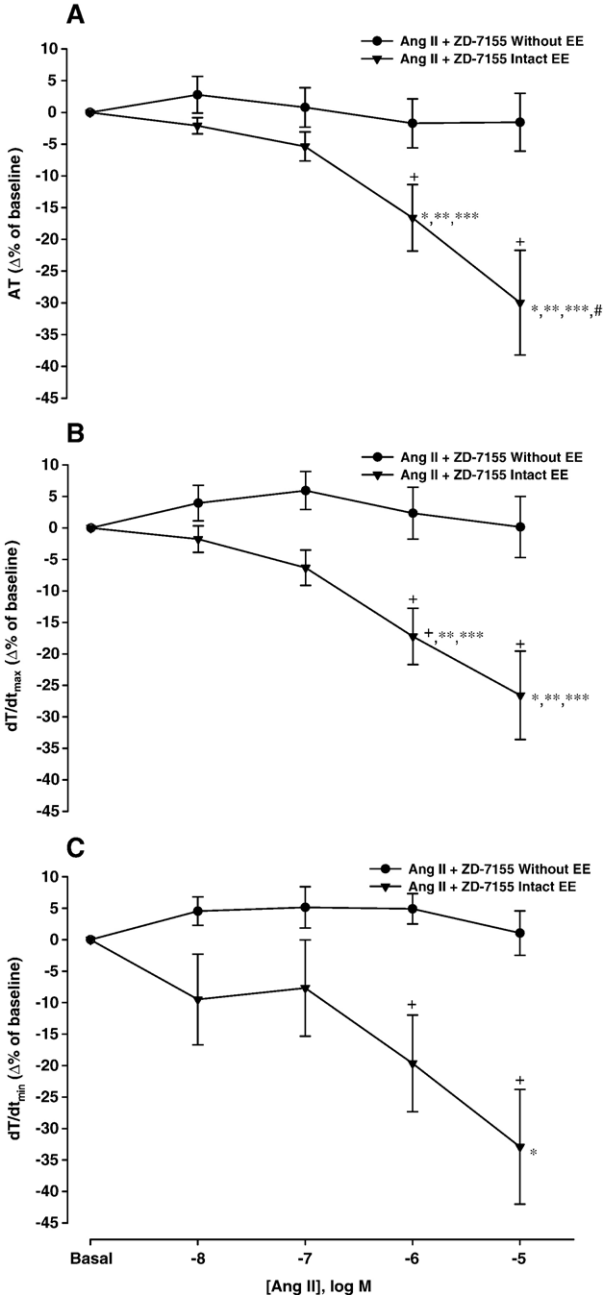

Fig. 2. Addition of increasing concentrations of angiotensin II in the presence of a selective antagonist of AT_1 receptors (ZD-7155) before and after selective removal of endocardial endothelium. The variables analyzed were active tension (AT; A) and peak rate of tension rise (dT/dt_{max}; B) or tension decline (dT/dt_{min}; C). *$P<0.05$ vs. basal; **$P<0.05$ vs. 10^{-8} M; ***$P<0.05$ vs. 10^{-7} M; #$P<0.05$ vs. 10^{-6} M; +$P<0.05$ vs. without endocardial endothelium.

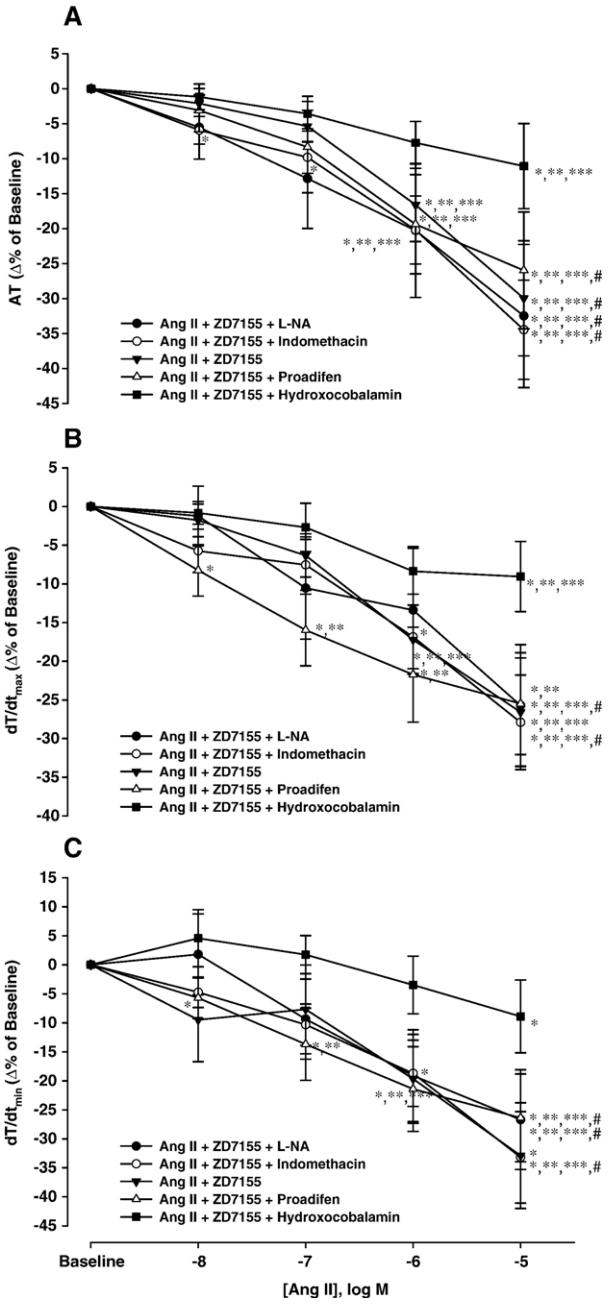

Fig. 3. Negative inotropic effect induced by the addition of increasing concentrations of angiotensin II in the presence of ZD-7155 and of NG-nitro-L-Arginine, indomethacin, proadifen or hydroxocobalamin. Selected parameters were active tension (AT; A) and peak rate of tension rise (dT/dt_{max}; B) and decline (dT/dt_{min}; C). *$P<0.05$ vs. basal; **$P<0.05$ vs. 10^{-8} M; ***$P<0.05$ vs. 10^{-7} M; #$P<0.05$ vs. 10^{-6} M.

In the following Protocols, we studied the mechanisms underlying the negative inotropic effect previously observed, by adding increasing concentrations of angiotensin II in presence of ZD-7155 in several experimental conditions.

The addition of increasing concentrations of angiotensin II in presence of ZD-7155 after the selective removal of endocardial endothelium abolished the negative inotropic effect previously observed (Fig. 2).

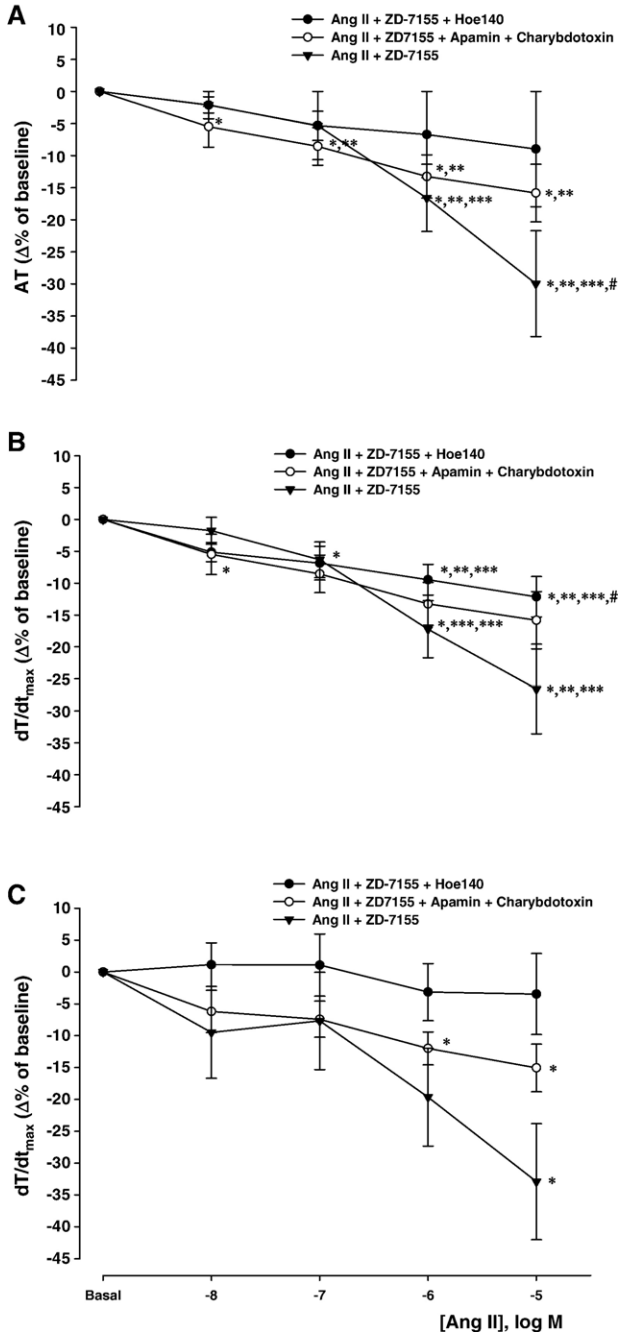

Fig. 4. Effects of increasing concentrations of angiotensin II plus ZD-7155 on active tension (AT; A) and peak rate of tension rise (dT/dt_{max}; B) or tension decline (dT/dt_{min}; C) in the absence and in the presence of Hoe-140, an antagonist of bradykinin B_2 receptors, and apamin plus charybdotoxin. *$P<0.05$ vs. basal; **$P<0.05$ vs. 10^{-8} M; ***$P<0.05$ vs. 10^{-7} M; #$P<0.05$ vs. 10^{-6} M.

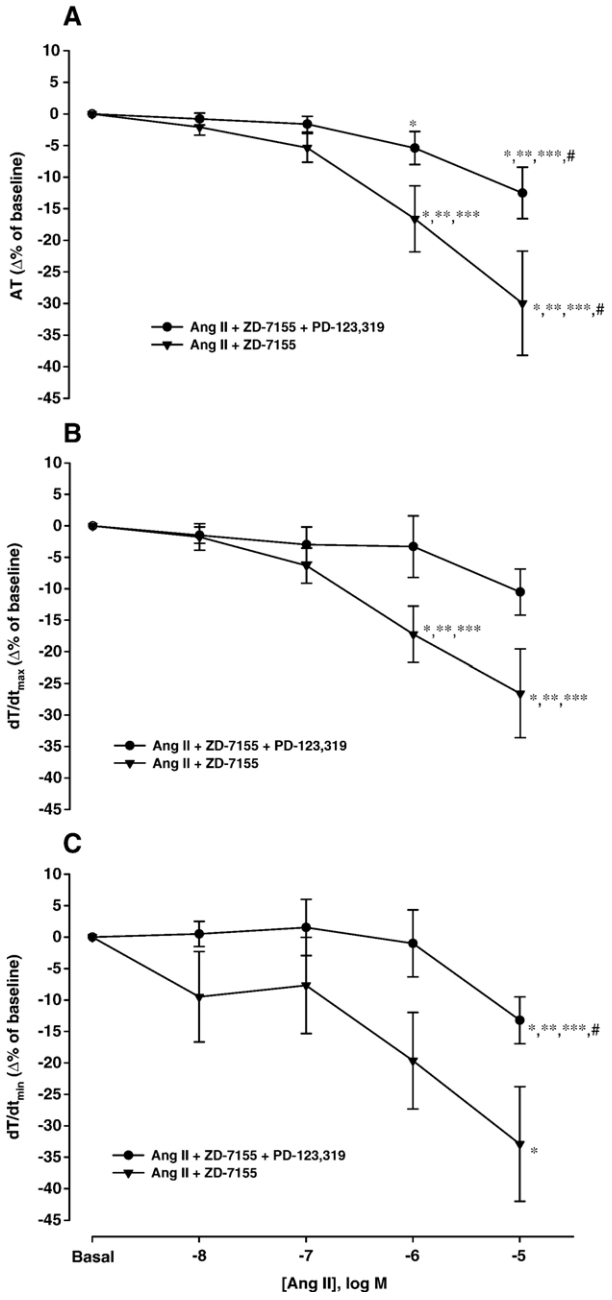

Fig. 5. Effects of increasing concentrations of angiotensin II plus ZD-7155 on active tension (AT; A) and peak rate of tension rise (dT/dt_{max}; B) or tension decline (dT/dt_{min}; C) in the absence and in the presence of PD-123,319, an antagonist of AT_2 receptors. *$P<0.05$ vs. basal; **$P<0.05$ vs. 10^{-8} M; ***$P<0.05$ vs. 10^{-7} M; #$P<0.05$ vs. 10^{-6} M.

The effects of angiotensin II in presence of ZD-7155 and inhibitors of the synthesis of nitric oxide, prostaglandins or endothelium-derived hyperpolarizing factor is shown in Fig. 3. The inhibitors used were, as previously stated, NG-nitro-L-Arginine, indomethacin and proadifen, respectively. In all three protocols, the negative inotropic effect was not significantly altered (Fig. 3). However, when angiotensin II was added in the presence of ZD-7155 and hydroxocobalamin (a nitric oxide scavenger) there was a significant attenuation of the negative inotropic effect. In fact, at the maximal concentration of

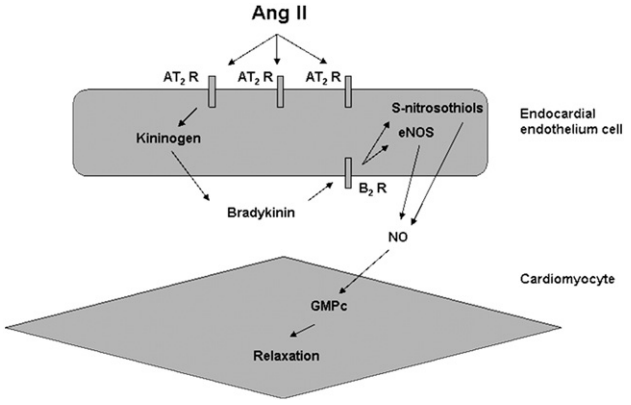

Fig. 6. Schematic diagram of the pathways involved in angiotensin AT_2 receptor activation. Ang II: angiotensin II; AT_2 R: angiotensin AT_2 receptor; B_2 R: bradykinin B_2 receptor; NO: nitric oxide; eNOS: constitutive nitric oxide synthase.

angiotensin II there was a decrease of only 11.1±6.1% in active tension, 9.1±4,5% in dT/dt_{max}, 8.9±6.3% in dT/dt_{min}, 10.5±4.5% in peak shortening and 13.3±3.2% in dL/dt_{max} (Fig. 3).

Fig. 4 compares the effects of the addition of angiotensin II in presence of ZD-7155 with or without an antagonist of B_2 bradykinin receptors, Hoe-140. As can be seen in the graph presented, the negative inotropic effect of angiotensin II in presence of ZD-7155 was significantly blunted at the higher concentration tested, decreasing 6.7±2.2% active tension, 9.5±2.4% dT/dt_{max}, 6.1±0.9% peak shortening and 6.7±1.1% dL/dt_{max}, without affecting dT/dt_{min}. The negative inotropic effect induced by angiotensin AT_2 stimulation was similarly blunted in the presence of charybdotoxin plus apamin, which block IK_{Ca}, BK_{Ca} and SK_{Ca} (Fig. 4).

The negative inotropic effect of the highest concentration of angiotensin II in presence of ZD-7155 was also blunted by the concomitant inhibition of angiotensin AT_2 receptors with PD-123,319, as can be seen in Fig. 5.

In what concerns myocardial relaxation, selective stimulation of angiotensin AT_2 receptors decreased dT/dt_{min} inducing a negative lusitropic effect, as seen in Fig. 1. However, it did not significantly alter myocardial resting length or passive tension and thus acute myocardial distensibility.

4. Discussion

In the present study, we demonstrated that the selective stimulation of rabbit myocardial angiotensin AT_2 receptors caused a negative inotropic and lusitropic effect.

This effect was observed both with an angiotensin AT_2 receptor agonist (H-9395) and after the addition of angiotensin II in presence of ZD-7155. The antagonist used in these protocols is a selective competitive angiotensin AT_1 receptor antagonist that is approximately ten times more potent than losartan in suppressing the angiotensin II induced pressor response (Junggren et al., 1996). The addition of H-9395 in the presence of ZD-7155 allowed us to increase the selectivity of the stimulation and, in fact, we observed a slight amplification of the negative inotropic effect, as shown in Fig. 1. In the two protocols using H-9395, the same magnitude of the effect was now observed for a concentration of the agonist ten times lower. On the contrary, and as expected, the negative inotropic effect secondary to angiotensin AT_2 receptors selective stimulation was blunted in the presence of PD-123,319, an angiotensin AT_2 receptor antagonist.

In what concerns the diastolic properties, selective angiotensin AT_2 receptor stimulation induced a decrease in the peak rate of tension decline (dT/dt_{min}), as can be seen in Fig. 1C, reflecting a slowing of myocardial relaxation and thus a negative lusitropic effect. However, when myocardial stiffness was analyzed, this parameter was not significantly altered. It is known from previous reports that a number of neurohumoral agents, like nitric oxide (Heymes et al., 1999; Shah et al., 1994), endothelin-1 (Leite-Moreira et al., 2003) and angiotensin II (Leite-Moreira et al., 2006), may acutely modulate myocardial distensibility. In particular, the effect of angiotensin II is mediated by angiotensin AT_1 receptor and protein kinase C activation (Leite-Moreira et al., 2006). The present work is concordant to these previous results since selective angiotensin AT_2 receptor stimulation did not seem to influence myocardial distensibility.

In order to evaluate the role of the endocardial endothelium, we selectively stimulated angiotensin AT_2 receptors before and after selective removal of endocardial endothelium. As shown in Fig. 2, the negative inotropic and lusitropic effect of angiotensin AT_2 receptor stimulation was completely abolished after endocardial endothelium removal. Cardiac endothelial cells, like all other endothelial cells, express and release a variety of auto-and paracrine agents, which directly influence cardiac metabolism, growth, contractile performance, and rhythmicity (Brutsaert, 2003). The synthesis, secretion, and activities of these endothelium-derived substances are closely linked.

It is known that the endothelium may be implicated in the mediation of some of angiotensin AT_2 receptor actions (Martin et al., 2006). In the vasculature, angiotensin AT_2 receptor stimulation induces an acute vasodilating effect. In the presence of an angiotensin AT_1 receptor antagonist, angiotensin II caused a 30% increase in the diameter of preconstricted, microperfused rabbit afferent and efferent arterioles in a PD-123,319-sensitive manner (Arima et al., 1997; Endo et al., 1997). This acute angiotensin AT_2 receptor-mediated vasodilator response may be endothelium-dependent or independent and appears to involve a range of signaling pathways, including nitric oxide and bradykinin production, activation of cytochrome P-450 epoxygenase pathways and modulation of K^+ channel activity (Widdop et al., 2003). Moreover, in the human heart, it has been recently shown that angiotensin AT_2 receptor stimulation in coronary microarteries induces endothelium-dependent vasodilation which is mediated by bradykinin B_2 receptors and nitric oxide (Batenburg et al., 2004a). Nitric oxide released either from cardiac endothelial cells or generated within cardiac myocytes can directly influence cardiac contractile function (Shah et al., 2000). Both endothelium-derived nitric oxide and exogenous nitric oxide donors induce an earlier onset of myocardial relaxation and/or reduce diastolic tone (Grocott-Mason et al.,

1994; Paulus et al., 1994). It has long been recognized that nitric oxide and prostaglandin I2 share a number of important properties and that their synthesis and release from endothelial cells are often coupled (Carter and Pearson, 1992). The endothelium is also an important mediator of chronic angiotensin AT_2 receptor actions. For instance, in a study performed by Wharton et al. (Wharton et al., 1998) the density of angiotensin AT_2 binding sites in endocardial, interstitial, and infarcted regions in the ventricles of patients with end-stage ischemic heart disease or dilated cardiomyopathy is significantly increased compared with the noninfarcted myocardium. And in a rat model of chronic heart failure, left ventricular remodeling and cardiac function were improved by blockade of angiotensin AT_1 receptors. This effect was inhibited by treatment with an angiotensin AT_2 receptor antagonist and also, in part, by treatment with a bradykinin B_2 receptor antagonist (Liu et al., 1997). In pigs, infarct size was reduced after regional myocardial ischemia by blockade of the angiotensin AT_1 receptor, and this reduction was abolished by pretreatment with the angiotensin AT_2 receptor antagonist PD-123,319 and by blockade of bradykinin B_2 receptors (Jalowy et al., 1998). The aforementioned reduction of perivascular fibrosis by overexpressed cardiac angiotensin AT_2 receptors after pressure overload was abolished after blockade of the bradykinin B_2 receptors or nitric oxide synthase. Thus, as in the vasculature, the myocardial kinin/nitric oxide system appears to be involved in angiotensin AT_2 receptor-mediated cardiac effects.

In order to further characterize the endothelium-dependent negative inotropic effect, we tested the effects of selective angiotensin AT_2 receptor stimulation in the presence of NG-nitro-L-Arginine, indomethacin, proadifen and hydroxocobalamin, inhibitors of the synthesis of nitric oxide, prostaglandins and endothelium-derived hyperpolarizing factor and a nitric oxide scavenger, respectively. We observed that in the presence of these substances, the magnitude of the negative inotropic and lusitropic effect was not significantly altered except with hydroxocobalamin, as seen in Fig. 3.

It is interesting that in the present protocols the inhibition of nitric oxide synthesis with NG-nitro-L-Arginine did not attenuate the negative inotropic effect. However, in the presence of a nitric oxide scavenger, hydroxocobalamin, the negative inotropic effect observed after selective angiotensin AT_2 receptor stimulation was blunted. It is known that nitric oxide synthase inhibitors, even at high concentrations, do not block nitric oxide release completely (Cohen et al., 1997). *In vivo* and *in vitro* studies (Dadisson et al., 1996; Danser et al., 1998) have shown that bradykinin also induces nitric oxide from stores of nitric oxide-containing factors, such as *S*-nitroso-thiols. Such sources become depleted only on repeated exposure to bradykinin or after prolonged nitric oxide synthase inhibition (Danser et al., 2000). A nitric oxide scavenger such as hydroxocobalamin may induce a more complete blockade of nitric oxide and thus may explain the inability of NG-nitro-L-Arginine to block the negative inotropic effect observed in the present work.

Although it may be too simplistic to try and define their properties independently from one another since many of these endothelium-derived agents modulate the actions of the other factors (Brutsaert, 2003), prostaglandins and endothelium-derived hyperpolarizing factor do not seem to be directly involved in the negative inotropic and lusitropic effect. This is in contrast to what happens with other vasodilating and negative inotropic pathways, like endothelin ET_B receptor stimulation. In fact, in the same animal species, the endothelium dependent ET_B-mediated negative inotropic effect is mediated by nitric oxide and prostaglandins (Leite-Moreira and Bras-Silva, 2004).

Finally, we tested the role of bradykinin B_2 receptors. In the presence of Hoe-140, an antagonist of bradykinin B_2 receptors, the negative inotropic and lusitropic effect of selective angiotensin AT_2 receptor stimulation was significantly blunted (Fig. 4). In the vasculature overexpression of angiotensin AT_2 receptors increases bradykinin production presumably by activating kininogenase(s) (Tsutsumi et al., 1998) and the inhibition of this enzyme might be another way to demonstrate an interaction between these two systems. Although this is true, our primary objective was to demonstrate that bradykinin B_2 receptors are involved in the mechanisms underlying angiotensin AT_2 receptor stimulation. There are several evidences in other organs that this interaction exists. In a study published by Bergaya et al., 2004, the authors examined the possible contribution of angiotensin AT_2 receptors to the kinin-dependent response to flow. They evaluated changes in outer diameter after increases in flow rate in perfused arteries from wild-type animals ($TK^{+}/^{+}$) and in tissue kallikrein-deficient mice ($TK^{-}/^{-}$) in which the presence of angiotensin AT_2 receptor expression was verified. Their results showed that if bradykinin B_2 receptors are blocked or if the vascular kinin–kallikrein system is inactivated, the angiotensin AT_2 receptor antagonist PD123319 no longer decreases the response to flow. Similarly, if angiotensin AT_2 receptors are blocked or not expressed, the bradykinin B_2 receptor antagonist Hoe-140 no longer inhibits flow-induced dilation. Our results are in accordance with these observations, since the blockade of bradykinin B_2 receptors with Hoe-140 attenuated the negative inotropic effect of angiotensin AT_2 receptor stimulation, thus suggesting that this effect depends on angiotensin AT_2 receptors stimulation and requires the presence of both functional bradykinin B_2 receptors and an active vascular kinin–kallikrein system. In the heart, there are also evidences linking these two systems. As shown by Kurisu et al., 2003, bradykinin released from cardiomyocytes through angiotensin AT_2 receptor signaling activates endothelial bradykinin B_2 receptors, leading to activation of constitutive nitric oxide synthas. They showed that this activation was followed by nitric oxide-dependent inhibition of fibrosis in perivascular fibroblasts. Our results suggest that these pathways are also involved in the modulation of the angiotensin AT_2 receptor dependent negative inotropic effect. This stresses the role of bradykinin B_2 receptors in the mediation of angiotensin AT_2 receptor actions in acute myocardial contractile parameters. Since it is known that prostaglandin I2 is a key mediator of bradykinin B_2 receptors effects (Ignarro et al., 1987), it is interesting that in the present protocols the inhibition of nitric oxide synthesis with indomethacin did not significantly alter myocardial contractile parameters.

The endothelium-dependent relaxation induced by bradykinin cannot fully be attributed to the release of nitric oxide. As demonstrated by Batenburg et al. in human coronary arteries there are other mechanisms of endothelial-dependent vasorelaxation induced by bradykinin B_2 receptor stimulation, such as the activation of a number of ion channels located in the smooth muscle cells that may account for "endothelium-dependent hyperpolarization" (Batenburg et al., 2004b). These channels are activated by factors other than nitric oxide and products of cytochrome P450 epoxygenase (Busse et al., 2002; Batenburg et al., 2004b). In the present study this was confirmed, since the angiotensin AT_2 receptor dependent negative inotropic effect was blunted in the presence of apamin and charybdotoxin, an IK_{Ca} and BK_{Ca} channel inhibitor and a SK_{Ca} channel inhibitor, respectively.

In conclusion, the renin angiotensin system has a central role in cardiovascular homeostasis both in healthy and non-healthy individuals. Indeed the pharmacological interventions modulating this neuro-humoral system are valuable tools in the treatment of hypertension, myocardial infarction, heart failure or renal failure. There are several ways of intervention within the renin angiotensin system, namely by inhibiting angiotensin converting enzyme or through the antagonization of angiotensin AT_1 receptors. In both of these interventions angiotensin AT_2 receptors seem to be responsible for at least some of the beneficial effects observed. The present study demonstrates, for the first time, that angiotensin AT_2 receptor stimulation induces a negative inotropic effect, thus clarifying the role of these receptors in myocardial contractility (Fig. 6). This effect was significantly blunted after the selective removal of endocardial endothelium or bradykinin B_2 receptor inhibition. This may add to the previous knowledge about the cardiac effects of angiotensin AT_2 receptor stimulation. Elucidation of the beneficial role of the angiotensin AT_2 in the human heart may contribute to the establishment of more sophisticated methods of treatment for human heart diseases.

Acknowledgements

Supported by Portuguese grants from FCT (POCTI/42517) through Unidade I&D Cardiovascular (51/94-FCT). There are no financial or other relations that could lead to a conflict of interest.

References

Arima, S., Endo, Y., Yaoita, H., Omata, K., Ogawa, S., Tsunoda, K., Abe, M., Takeuchi, K., Abe, K., Ito, S., 1997. Possible role of P-450 metabolite of arachidonic acid in vasodilator mechanism of angiotensin II type 2 receptor in the isolated microperfused rabbit afferent arteriole. J. Clin. Invest. 100, 2816–2823.

Bartunek, J., Weinberg, E.O., Tajima, M., Rohrbach, S., Lorell, B.H., 1999. Angiotensin II type 2 receptor blockade amplifies the early signals of cardiac growth response to angiotensin II in hypertrophied hearts. Circulation 99, 22–25.

Batenburg, W.W., Garrelds, I.M., Bernasconi, C.C., Juillerat-Jeanneret, L., van Kats, J.P., Saxena, P.R., Danser, A.H., 2004a. Angiotensin II type 2 receptor-mediated vasodilation in human coronary microarteries. Circulation 109, 2296–2301.

Batenburg, W.W., Garrelds, I.M., van Kats, J.P., Saxena, P.R., Danser, A.H., 2004b. Mediators of bradykinin-induced vasorelaxation in human coronary microarteries. Hypertension 43, 488–492.

Bergaya, S., Hilgers, R.H., Meneton, P., Dong, Y., Bloch-Faure, M., Inagami, T., Alhenc-Gelas, F., Lévy, B.I., Boulanger, C.M., 2004. Flow-dependent dilation mediated by endogenous kinins requires angiotensin AT_2 receptors. Circ. Res 25, 1623–1629.

Berges, A., Van Nassauw, L., Timmermans, J.P., Vrints, C., 2005. Role of nitric oxide during coronary endothelial dysfunction after myocardial infarction. Eur. J. Pharmacol. 516, 60–70.

Booz, G.W., Baker, K.M., 1996. Role of type 1 and type 2 angiotensin receptors in angiotensin II–induced cardiomyocyte hypertrophy. Hypertension 28, 635–640.

Brink, M., de Gasparo, M., Rogg, H., Whitebread, S., Bullock, G., 1995. Localization of angiotensin II receptor subtypes in the rabbit heart. J. Mol. Cell. Cardiol. 27, 459–470.

Brutsaert, D.L., 2003. Cardiac endothelial-myocardial signaling: its role in cardiac growth, contractile performance, and rhythmicity. Physiol. Rev. 83, 59–115.

Brutsaert, D.L., Meulemans, A.L., Spido, K.R., Sys, S.U., 1988. Effects of damaging endocardial surface on the mechanical performance of isolated cardiac muscle. Circ. Res. 62, 358–366.

Busche, S., Gallinat, S., Bohle, R.M., Reinecke, A., Seebeck, J., Franke, F., Fink, L., Zhu, M., Sumners, C., Unger, T., 2000. Expression of angiotensin AT(1) and AT(2) receptors in adult rat cardiomyocytes after myocardial infarction. A single-cell reverse transcriptase-polymerase chain reaction study. Am. J. Pathol. 157, 605–611.

Busse, R., Edwards, G., Feletou, M., Fleming, I., Vanhoutte, P.M., Weston, A.H., 2002. EDHF: bringing the concepts together. Trends Pharmacol. Sci. 23, 374–380.

Carter, T.D., Pearson, J.D., 1992. Regulation of prostacyclin synthesis in endothelial cells. NIPS 7, 64–69.

Chang, R.S., Lotti, V.J., 1991. Angiotensin receptor subtypes in rat, rabbit and monkey tissues: relative distribution and species dependency. Life Sci. 49, 1485–1490.

Chiu, A.T., Herblin, W.F., McCall, D.E., Ardecky, R.J., Carini, D.J., Duncia, J.V., Pease, L.J., Wong, P.C., Wexler, R.R., Johnson, A.L., et al., 1989. Identification of angiotensin II receptor subtypes. Biochem. Biophys. Res. Commun. 165, 196–203.

Cohen, R.A., Plane, F., Najibi, S., Huk, I., Malinski, T., Garland, C.J., 1997. Nitric oxide is the mediator of both endothelium-dependent relaxation and hyperpolarization of the rabbit carotid artery. Proc. Natl. Acad. Sci. U.S.A 94, 4193–4198.

Dadisson, R.L., Bates, J.N., Johnson, K., Lewis, S.J., 1996. Use-dependent loss of acetylcholine-and bradykinin-mediated vasodilation after nitric oxide synthase inhibition. Evidence for preformed stores of nitric oxide-containing factors in vascular endothelial cells. Hypertension 28, 354–360.

Danser, A.H.J., De Vries, R., Schoemaker, R.G., Saxena, P.R., 1998. Bradykinin-induced release of nitric oxide by the isolated perfused rat heart: importance of preformed pools of nitric oxide-containing factors. J. Hypertens. 16, 239–244.

Danser, A.H.J., Tom, B., de Vries, R., Saxena, P.R., 2000. L-NAME resistant bradykinin-induced relaxation in porcine coronary arteries is NO-dependent: effect of ACE inhibition. Br J Pharmacol. 131, 195–202.

de Gasparo, M., Catt, K.J., Inagami, T., Wright, J.W., Unger, T., 2000. International union of pharmacology. XXIII. The angiotensin II receptors. Pharmacol. Rev. 52, 415–472.

Endo, Y., Arima, S., Yaoita, H., Omata, K., Tsunoda, K., Takeuchi, K., Abe, K., Ito, S., 1997. Function of angiotensin II type 2 receptor in the postglomerular efferent arteriole Kidney Int. Suppl. 63, S205–S207.

Gallinat, S., Busche, S., Raizada, M.K., Sumners, C., 2000. The angiotensin II type 2 receptor: an enigma with multiple variations. Am. J. Physiol. Endocrinol. Metab. 278, E357–E374.

Grocott-Mason, R., Fort, S., Lewis, M.J., Shah, A.M., 1994. Myocardial relaxant effect of exogenous nitric oxide in the isolated ejecting heart. Am. J. Physiol. Heart Circ. Physiol. 266, H1699–H1705.

Haberl, R.L., 1994. Role of angiotensin receptor subtypes in the response of rabbit brain arterioles to angiotensin. Stroke 25, 1476–1479.

Heymes, C., Vanderheyden, M., Bronzwaer, J.G., Shah, A.M., Paulus, W.J., 1999. Endomyocardial nitric oxide synthase and left ventricular preload reserve in dilated cardiomyopathy. Circulation 99, 3009–3016.

Horiuchi, M., Akishita, M., Dzau, V.J., 1999. Recent progress in angiotensin II type 2 receptor research in the cardiovascular system. Hypertension 33, 613–621.

Ignarro, L.J., Byrns, R.E., Buga, G.M., Wood, K.S., 1987. Mechanisms of endothelium dependent vascular smooth muscle relaxation elicited by bradykinin and VIP. Am. J. Physiol. 253, H1074–H1082.

Jalowy, A., Schulz, R., Dorge, H., Behrends, M., Heusch, G., 1998. Infarct size reduction by AT1-receptor blockade through a signal cascade of AT_2-receptor activation, bradykinin and prostaglandins in pigs. J. Am. Coll. Cardiol. 32, 1787–1796.

Johren, O., Dendorfer, A., Dominiak, P., 2004. Cardiovascular and renal function of angiotensin II type-2 receptors. Cardiovasc. Res. 62, 460–467.

Junggren, I.L., Zhao, X., Sun, X., Hedner, T., 1996. Comparative cardiovascular effects of the angiotensin II type 1 receptor antagonists ZD 7155 and losartan in the rat. J. Pharm. Pharmacol. 48, 829–833.

Kato, M., Nishida, S., Kitasato, H., Sakata, N., Kawai, S., 2001. Cyclooxygenase-1 and cyclooxygenase-2 selectivity of non-steroidal anti-inflammatory drugs: investigation using human peripheral monocytes. J. Pharm. Pharmacol. 53, 1679–1685.

Kurisu, S., Ozono, R., Oshima, T., Kambe, M., Ishida, T., Sugino, H., Matsuura, H., Chayama, K., Teranishi, Y., Iba, O., Amano, K., Matsubara, H., 2003. Cardiac angiotensin II type 2 receptor activates the kinin/NO system and inhibits fibrosis. Hypertension 41, 99–107.

Leite-Moreira, A.F., Bras-Silva, C., 2004. Inotropic effects of ETB receptor stimulation and their modulation by endocardial endothelium, NO, and prostaglandins. Am. J. Physiol. Heart Circ. Physiol. 287, H1194–H1199.

Leite-Moreira, A., Brás-Silva, C., Pedrosa, C., Rocha-Sousa, A., 2003. ET-1 increases distensibility of acutely loaded myocardium: a novel ETA and Na+/H+exchanger effect. Am. J. Physiol. Heart Circ. Physiol. 284, H1332–H1339.

Leite-Moreira, A.F., Castro-Chaves, P., Pimentel-Nunes, P., Lima-Carneiro, A., Guerra, M.S., Soares, J.B., Ferreira-Martins, J., 2006. Angiotensin II acutely decreases myocardial stiffness: a novel AT_1, PKC and Na+/H+exchanger-mediated effect. Br. J. Pharmacol. 147, 690–697.

Liu, Y.H., Yang, X.P., Sharov, V.G., Nass, O., Sabbah, H.N., Peterson, E., Carretero, O.A., 1997. Effects of angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor antagonists in rats with heart failure. Role of kinins and angiotensin II type 2 receptors. J. Clin. Invest. 99, 1926–1935.

Lopez, J.J., Lorell, B.H., Ingelfinger, J.R., Weinberg, E.O., Schunkert, H., Diamant, D., Tang, S.S., 1994. Distribution and function of cardiac angiotensin AT_1-and AT_2-receptor subtypes in hypertrophied rat hearts. Am. J. Physiol. 267, H844–H852.

Martin, P., Meher, A.P., Kreutz, R., 2006. Physiology of local renin-angiotensin systems. Physiol. Rev. 86, 747–803.

Matsubara, H., 1998. Pathophysiological role of angiotensin II type 2 receptor in cardiovascular and renal diseases. Circ. Res. 83, 1182–1191.

Mohan, P., Brutsaert, D.L., Sys, S.U., 1995. Myocardial performance is modulated by interaction of cardiac endothelium derived nitric oxide and prostaglandins. Cardiovasc. Res. 29, 637–640.

Nakajima, M., Hutchinson, H.G., Fujinaga, M., Hayashida, W., Morishita, R., Zhang, L., Horiuchi, M., Pratt, R.E., Dzau, V.J., 1995. The angiotensin II type 2 (AT_2) receptor antagonizes the growth effects of the AT_1 receptor: gain-of-function study using gene transfer. Proc. Natl. Acad. Sci. U. S. A. 92, 10663–10667.

Ohkubo, N., Matsubara, H., Nozawa, Y., Mori, Y., Murasawa, S., Kijima, K., Maruyama, K., Masaki, H., Tsutumi, Y., Shibazaki, Y., Iwasaka, T., Inada, M., 1997. Angiotensin type 2 receptors are reexpressed by cardiac fibroblasts from failing myopathic hamster hearts and inhibit cell growth and fibrillar collagen metabolism. Circulation 96, 3954–3962.

Paulus, W.J., Vantrimpont, P.J., Shah, A.M., 1994. Acute effects of nitric oxide on left ventricular relaxation and diastolic distensibility in humans. Circulation 89, 2070–2078.

Regitz-Zagrosek, V., Friedel, N., Heymann, A., Bauer, P., Neuss, M., Rolfs, A., Steffen, C., Hildebrandt, A., Hetzer, R., Fleck, E., 1995. Regulation, chamber localization, and subtype distribution of angiotensin II receptors in human hearts. Circulation 91, 1461–1471.

Schneider, M.D., Lorell, B.H., 2001. AT(2), judgment day: which angiotensin receptor is the culprit in cardiac hypertrophy? Circulation 104, 247–248.

Shah, A.M., MacCarthy, P.A., 2000. Paracrine and autocrine effects of nitric oxide on myocardial function. Pharmacol. Ther. 86, 49–86.

Shah, A.M., Spurgeon, H.A., Sollot, S.J., Talo, A., Lakatta, E.G., 1994. 8-bromo-cGMP reduces the myofilament response to $Ca2^+$ in intact cardiac myocytes. Circ. Res. 74, 970–978.

Tsutsumi, Y., Matsubara, H., Ohkubo, N., Mori, N., Nozawa, Y., Murasawa, S., Kijima, K., Maruyama, K., Masaki, H., Moriguchi, Y., Shibasaki, Y., Kamihata, H., Inada, M., Iwasaka, T., 1998. Angiotensin II type 2 receptor is upregulated in human heart with interstitial fibrosis, and cardiac fibroblasts are the major cell type for its expression. Circ. Res. 83, 1035–1046.

Wharton, J., Morgan, K., Rutherford, R.A., Catravas, J.D., Chester, A., Whitehead, B.F., De Leval, M.R., Yacoub, M.H., Polak, J.M., 1998. Differential distribution of angiotensin AT_2 receptors in the normal and failing human heart. J. Pharmacol. Exp. Ther. 284, 323–336.

Whitebread, S., Mele, M., Kamber, B., de Gasparo, M., 1989. Preliminary biochemical characterization of two angiotensin II receptor subtypes. Biochem. Biophys. Res. Commun. 163, 284–291.

Widdop, R.E., Jones, E.S., Hannan, R.E., Gaspari, T.A., 2003. Angiotensin AT_2 receptors: cardiovascular hope or hype? Br. J. Pharmacol. 140, 809–824.

Widdop, R.E., Matrougui, K., Levy, B.I., Henrion, D., 2002. AT2 receptor-mediated relaxation is preserved after long-term AT_1 receptor blockade. Hypertension 40, 516–520.