Negative inotropic effect of selective AT$_2$ receptor stimulation and its modulation by the endocardial endothelium

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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.

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

In fetal tissues, angiotensin AT₂ receptor is the predominant subtype expressed. After birth, its expression in mesenchimal tissues rapidly decreases. The common misconception that angiotensin AT₂ 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 AT₂ 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 compared with controls (Lopez et al., 1994; Ohkubo et al., 1997; Bartunek et al., 1999). In rabbit hearts, this receptor subtype is 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 compared with controls (Lopez et al., 1994; Ohkubo et al., 1997; Bartunek et al., 1999). In rat model of chronic heart failure, left ventricular remodeling and function are improved by blockade of angiotensin AT1 receptors (Scheider and Lorell, 2001).

2. Materials and methods


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, MgSO₄ 2.4, KH₂PO₄ 1.2, CaCl₂ 1.8, NaHCO₃ 20, CH₃COONa 5, C₆H₁₂O₄Na 15, glucose 4.5 and atenolol 0.02. Atenolol was used to prevent β-adrenergic mediated effects. The solutions were in equilibrium with 95% O₂ and 5% CO₂, 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 interval.

2.2. Experimental protocols

In a first set of protocols, we studied the effects of selective angiotensin AT₂ receptor stimulation. H-9395 (Nicotinoyl-Tyr-Lys(Z-Arg)-His-Pro-Ile-OH, supplied from Bachem AG), an agonist of angiotensin AT₂ 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 AT₁ 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 AT₁ receptor antagonist (ZD7155, 10⁻⁶ M; n=12). ZD-7155 is an angiotensin AT₁ 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 AT₂ receptor stimulation. This was performed by adding increasing concentrations of angiotensin II (10⁻⁸, 10⁻⁷, 10⁻⁶, 10⁻⁵ M) in the absence or presence of a selective angiotensin AT₁ receptor antagonist (ZD7155, 10⁻⁶ M; n=12). ZD-7155 is an angiotensin AT₁ receptor competitive antagonist that is approximately ten times more potent than losartan in suppressing the angiotensin II-induced pressor response (Junggren et al., 1996).
10⁻⁶, 10⁻⁵ M) in presence of ZD-7155 (5,7-Diethyl-3,4-dihydro-1-[(2,1'-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 (3-(Arg-l-Arg-l-Pro-l-Hyp-Gly-l-(2-thienyl)Ala-l-Ser-l-D-1,2,3,4-tetrahydro-3-isoquinolinecarbonyl-l-(2α,3β,7αβ)-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 B₂ receptors, a nitric oxide scavenger, an IKCa channel inhibitor and a SKCa 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 AT₂ 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/dtmax, mN mm⁻² s⁻¹); maximum velocity of tension decline (dT/dtmin, mN mm⁻² s⁻¹); peak isotonic shortening (%Lmax); maximum velocity of shortening (dL/dtmax, Lmax s⁻¹); maximum velocity of lengthening (dL/dtmin, Lmax 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/mm²; dT/dtmax 185±15 mN/mm² s; dT/dtmin 161±13 mN/mm² s; peak shortening 10±0.8% of Lmax; dL/dtmax 0.72±0.04 Lmax s⁻¹; dL/dtmin 2.4±0.17 Lmax 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/dtmax; B) or tension decline (dT/dtmin; C). On the axis, [X] represents the concentrations of angiotensin II or H-9395, according to the respective protocol. Selective stimulation of AT₂ receptors induced negative inotropic and lusitropic effects maximal for the concentration of 10⁻⁷ 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 AT2 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 $16.4\pm6.8\%$ active tension, $20.3\pm6.0\%$ $\frac{dT}{dt_{\text{max}}}$, $19.6\pm7.3\%$ $\frac{dT}{dt_{\text{min}}}$, $16.0\pm5.6\%$ peak shortening and $32.3\pm12.2\%$ $\frac{DL}{dt_{\text{max}}}$ ($P<0.05$). The addition of the same agonist in the presence of a selective angiotensin AT1 receptor antagonist (ZD-7155) increased the selectivity of the stimulation and caused a slight amplification of the negative inotropic effect (Fig. 1).

![Fig. 1](image1.png)

**Fig. 1.** 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 ($\frac{dT}{dt_{\text{max}}}$; B) and decline ($\frac{dT}{dt_{\text{min}}}$; C). *$P<0.05$ vs. basal; **$P<0.05$ vs. $10^{-8}$ M; ***$P<0.05$ vs. $10^{-7}$ M; #*$P<0.05$ vs. without endocardial endothelium.

![Fig. 2](image2.png)

**Fig. 2.** Addition of increasing concentrations of angiotensin II in the presence of a selective antagonist of AT1 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 ($\frac{dT}{dt_{\text{max}}}$; B) or tension decline ($\frac{dT}{dt_{\text{min}}}$; C). *$P<0.05$ vs. basal; **$P<0.05$ vs. $10^{-8}$ M; ***$P<0.05$ vs. $10^{-7}$ M; #*$P<0.05$ vs. without endocardial endothelium.

![Fig. 3](image3.png)

**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 ($\frac{dT}{dt_{\text{max}}}$; B) and decline ($\frac{dT}{dt_{\text{min}}}$; C). *$P<0.05$ vs. basal; **$P<0.05$ vs. $10^{-8}$ M; ***$P<0.05$ vs. $10^{-7}$ M; #*$P<0.05$ vs. without endocardial endothelium.

The stimulation and caused a slight amplification of the negative inotropic effect (Fig. 1). Selective angiotensin AT2 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% $\frac{dT}{dt_{\text{max}}}$, 32.9±9.1% $\frac{dT}{dt_{\text{min}}}$, 26.8±7.0% peak shortening and 22.4±5.8% $\frac{DL}{dt_{\text{max}}}$.

This effect was not significantly different from the one observed with the addition of H-9395 in the presence of ZD-7155.
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).

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. 4. Effects of increasing concentrations of angiotensin II plus ZD-7155 on active tension (AT; A) and peak rate of tension rise (dAT/dt\text{max}; B) or tension decline (dAT/dt\text{min}; C) in the absence and in the presence of Hoe-140, an antagonist of bradykinin B\text{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 (dAT/dt\text{max}; B) or tension decline (dAT/dt\text{min}; C) in the absence and in the presence of PD-123,319, an antagonist of AT\text{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
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).

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., 2000).
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 AT2 receptor actions. For instance, in a study performed by Wharton et al. (Wharton et al., 1998) the density of angiotensin AT2 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 AT1 receptors. This effect was inhibited by treatment with an angiotensin AT2 receptor antagonist and also, in part, by treatment with a bradykinin B2 receptor antagonist (Liu et al., 1997). In pigs, infarct size was reduced after regional myocardial ischemia by blockade of the angiotensin AT1 receptor, and this reduction was abolished by pretreatment with the angiotensin AT2 receptor antagonist PD-123,319 and by blockade of bradykinin B2 receptors (Jalowy et al., 1998). The aforementioned reduction of perivascular fibrosis by overexpressed cardiac angiotensin AT2 receptors after pressure overload was abolished after blockade of the bradykinin B2 receptors or nitric oxide synthase. Thus, as in the vasculature, the myocardial kinin/nitric oxide system appears to be involved in angiotensin AT2 receptor-mediated cardiac effects.

In order to further characterize the endothelium-dependent negative inotropic effect, we tested the effects of selective angiotensin AT2 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 AT2 receptor stimulation was blunted. It is known that nitric oxide synthase inhibitors, even at high concentrations, do not block 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 ETB receptor stimulation. In fact, in the same animal species, the endothelium dependent ETB-mediated negative inotropic effect is mediated by nitric oxide and prostaglandins (Leite-Moreira and Brás-Silva, 2004).

Finally, we tested the role of bradykinin B2 receptors. In the presence of Hoe-140, an antagonist of bradykinin B2 receptors, the negative inotropic and lusitropic effect of selective angiotensin AT2 receptor stimulation was significantly blunted (Fig. 4). In the vasculature overexpression of angiotensin AT2 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 B2 receptors are involved in the mechanisms underlying angiotensin AT2 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 AT2 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 AT2 receptor expression was verified. Their results showed that if bradykinin B2 receptors are blocked or if the vascular kinin–kallikrein system is inactivated, the angiotensin AT2 receptor antagonist PD123319 no longer decreases the response to flow. Similarly, if angiotensin AT2 receptors are blocked or not expressed, the bradykinin B2 receptor antagonist Hoe-140 no longer inhibits flow-induced dilation. Our results are in accordance with these observations, since the blockade of bradykinin B2 receptors with Hoe-140 attenuated the negative inotropic effect of angiotensin AT2 receptor stimulation, thus suggesting that this effect depends on angiotensin AT2 receptors stimulation and requires the presence of both functional bradykinin B2 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 AT2 receptor signaling activates endothelial bradykinin B2 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 AT2 receptor dependent negative inotropic effect. This stresses the role of bradykinin B2 receptors in the mediation of angiotensin AT2 receptor actions in acute myocardial contractile parameters. Since it is known that prostaglandin I2 is a key mediator of bradykinin B2 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 B2 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 AT2 receptor dependent negative inotropic effect was blunted in the presence of apamin and charybdotoxin, an IKCa and BKCa channel inhibitor and a SKCa 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 AT1 receptors. In both of these interventions angiotensin AT2 receptors seem to be responsible for at least some of the beneficial effects observed. The present study demonstrates, for the first time, that angiotensin AT2 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 B2 receptor inhibition. This may add to the previous knowledge about the cardiac effects of angiotensin AT2 receptor stimulation. Elucidation of the beneficial role of the angiotensin AT2 in the human heart may contribute to the establishment of more sophisticated methods of treatment for human heart diseases.

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