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AT_2 receptor-mediated vasodilation in the mouse heart depends on AT_{1A} receptor activation

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1 Angiotensin (Ang) II type 2 (AT_2) receptors are believed to counteract Ang II type 1 (AT_1) receptor-mediated effects. Here, we investigated AT_2 receptor-mediated effects on coronary and cardiac contractility in C57BL/6 mice.

2 Hearts were perfused according to Langendorff. Baseline coronary flow (CF) and left ventricular systolic pressure (LVSP) were 2.7 ± 0.1 ml min^{-1} and 111 ± 3 mmHg ($n = 50$), respectively.

3 Ang II ($n = 14$) concentration dependently decreased CF and LVSP, by maximally 41 ± 4 and $25 \pm 3\%$, respectively (pEC_{50}s 7.41 ± 0.12 and 7.65 ± 0.12). The AT_1 receptor antagonist irbesartan ($n = 4$) abolished all Ang II-induced changes, whereas the AT_2 receptor antagonist PD123319 ($n = 6$) enhanced ($P < 0.05$) the effect of Ang II on CF (to $59 \pm 1\%$) and LVSP (to $44 \pm 2\%$), without altering its potency. A similar enhancement was observed in the presence of nitric oxide (NO) synthase inhibitor N^{ω}-nitro-L-arginine methyl ester HCl (L-NAME; $n = 4$). On top of L-NAME, PD123319 no longer affected the response to Ang II ($n = 4$).

4 The AT_2 receptor agonist CGP42112A ($n = 4$) did not affect CF or LVSP, nor did CGP42112A ($n = 4$) alter the constrictor response to the α_1-adrenoceptor agonist phenylephrine. Furthermore, Ang II exerted no effects in hearts of $AT_{1A}^{-/-}$ mice ($n = 5$), whereas its effects in hearts of $AT_{1A}^{+/+}$ wild-type control mice ($n = 7$) were indistinguishable from those in hearts of C57BL/6 mice.

5 In conclusion, Ang II exerts opposite effects on coronary and cardiac contractility in the mouse heart *via* activation of AT_{1A} and AT_2 receptors. AT_2 receptor-mediated effects depend on NO and occur only in conjunction with AT_{1A} receptor activation.
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Abbreviations: Ang, angiotensin; AT_1 receptor, angiotensin II type 1 receptor; AT_2 receptor, angiotensin II type 2 receptor; CF, coronary flow; KH, Krebs–Henseleit; L-NAME, N^{ω}-nitro-L-arginine methyl ester HCl; LVSP, left ventricular systolic pressure; NO, nitric oxide; NOS, nitric oxide synthase; RAS, renin–angiotensin system

Introduction

The renin–angiotensin system (RAS) plays an important role in the regulation of blood pressure, cardiovascular remodeling and maintaining body fluid volume. The main effector peptide of the RAS, angiotensin (Ang) II, activates Ang II type 1 (AT_1) and Ang II type 2 (AT_2) receptors. Two subtypes of AT_1 receptor have been identified in rodents (AT_{1A} and AT_{1B}) which share 94% sequence homology, whereas the AT_2 receptor only shares 34% sequence homology with these subtypes (Elton *et al*., 1992; Iwai & Inagami, 1992; Mukoyama *et al*., 1993). AT_1 receptors mediate the well-known vasoconstrictor, inotropic, chronotropic, aldosterone-releasing, noradrenaline-releasing and growth-stimulatory effects of Ang II, and AT_2 receptors are generally assumed to counteract these actions (Hein *et al*., 1995; Ichiki *et al*., 1995; Munzenmaier & Greene, 1996; van Kesteren *et al*., 1997; Masaki *et al*., 1998; Siragy *et al*., 1999; Schuijt *et al*., 2001; Batenburg *et al*., 2004). It is believed that AT_2 receptor-mediated vasodilation is an endothelium-dependent phenomenon, involving bradykinin type 2 receptors, nitric oxide (NO) and guanosine cyclic 3′, 5′-monophosphate (Siragy & Carey, 1997; Tsutsumi *et al*., 1999; Katada & Majima, 2002; Hannan *et al*., 2003; Batenburg *et al*., 2005).

However, not all studies confirm the counter-regulatory actions of AT_2 receptors (Levy *et al*., 1996; Ichihara *et al*., 2001; Duke *et al*., 2005; You *et al*., 2005). Findings on AT_2 receptor-mediated effects that contrasted with the above concept have been attributed to disparities in genetic background (Schneider & Lorell, 2001) or blood pressure (You *et al*., 2005). Furthermore, in many studies, conclusions on AT_2 receptor-mediated counteracting effects were drawn based on indirect evidence, that is, the occurrence of an enhanced response to Ang II following AT_2 receptor antagonism or gene disruption (Hein *et al*., 1995; Ichiki *et al*., 1995; Schuijt *et al*., 2001; Hannan *et al*., 2003). The interpretation of data obtained in the absence of AT_2 receptors is complex, because AT_2 receptors downregulate AT_1 receptors in a ligand-independent manner (Jin *et al*., 2002), and AT_2 receptor-null mice display increased AT_1 receptor expression (Tanaka *et al*., 1999).

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In the present study, we set out to characterize AT_2 receptor-mediated effects in the coronary vascular bed of the mouse heart using the Langendorff model. Despite the many AT_2 receptor-related studies in transgenic mice, such data are currently not available. AT_2 receptor-mediated responses were studied by comparing Ang II-induced responses in the absence and presence of the AT_2 receptor antagonist PD123319, and by selectively stimulating AT_2 receptors. The latter was accomplished in three ways. First, we investigated the effects of Ang II in the presence of the AT_1 receptor antagonist irbesartan. Second, we studied the effects of the AT_2 receptor agonist CGP42112A (Yee *et al*., 1998; Barber *et al*., 1999; Li & Widdop, 2004), both at baseline and during phenylephrine-induced vasoconstriction. Third, we evaluated the response to Ang II in $AT_{1A}^{-/-}$ mice, that is, mice lacking Ang II-induced vasoconstriction (Ito *et al*., 1995). We also investigated whether NO mediated the AT_2 receptor-dependent responses using the NO synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester HCl (L-NAME).

Methods

Animals

Male C57BL/6 mice (26 ± 0.6 g; $n = 50$) were obtained from Harlan (Zeist, The Netherlands). Male $AT_{1A}^{+/+}$ (31 ± 1 g; $n = 7$) and $AT_{1A}^{-/-}$ mice (27 ± 2 g; $n = 5$) were bred on a $129 \times$ C57BL/6 background at the animal facilities of the Charité, Campus Benjamin Franklin, Berlin, Germany (Oliverio *et al*., 1997). All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC, Rotterdam, The Netherlands.

Drugs

Ang II, CGP42112A, PD123319, bradykinin, endothelin-1, phenylephrine and L-NAME were purchased from Sigma (Zwijndrecht, The Netherlands). Irbesartan was a kind gift of Sanofi-Synthelabo BV (Gouda, The Netherlands). Irbesartan (10 mM) was dissolved in ethanol whereas all other chemicals were dissolved in water. Stock solutions were stored in aliquots at -80°C and diluted in modified Krebs–Henseleit (KH) perfusion buffer (composition in mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.2, D-glucose 11, $NaHCO_3$ 25 and pyruvic acid 2) on the day of the experiment. All perfusion solutions were passed through a 0.46 μm cellulose acetate filter (Millipore, Billerica, MA, U.S.A.) before their application in the Langendorff setup.

Langendorff preparation

Mice were heparinized using heparin (200 IU; i.p.) and subsequently killed by cervical dislocation (Gustafson & van Beek, 2000). The heart was rapidly excised and placed in ice-cold modified KH buffer, gassed with 95% O_2 and 5% CO_2 (Gustafson & van Beek, 2000; Wang *et al*., 2002). The aorta was immediately cannulated with a 19G needle (with a small circumferential grove close to the blunt tip) and perfused with gassed KH buffer according to Langendorff at a constant perfusion pressure of 80 mmHg (Sutherland *et al*., 2003). Two needle electrodes were placed at the right atrium and the hearts were paced at ~ 600 b.p.m. (5 Hz, 4 min duration, 4 V) using a Grass stimulator (Grass Instruments Co., Quincy, MA, U.S.A.).

Left ventricular systolic pressure (LVSP) was measured with a water-filled balloon (made of domestic food wrap) connected to a disposable pressure transducer (Braun, Melsungen, Germany). The left atrium was removed and the balloon was inserted into the left ventricle (Curtis *et al*., 1986; Sutherland *et al*., 2003). The left ventricular end-diastolic pressure was set at 3–5 mmHg by adjusting the balloon volume. Coronary flow (CF) was measured with a flow probe (Transonic systems, Ithaca, NY, U.S.A.).

Experimental protocol

After a stabilization period of 10–15 min, baseline values of CF and LVSP were obtained. Next, bolus injections (100 μl) of modified KH buffer were applied three times to determine injection-induced changes in CF and LVSP. Subsequently, bolus injections (100 μl) of Ang II, CGP42112A, bradykinin or endothelin-1 (concentration range in the injection fluid 0.1 nM–0.1 mM) were applied, in the absence or presence of irbesartan (1 μM in the perfusion buffer), PD123319 (1 μM) and/or the NOS inhibitor L-NAME (10 mM). All blockers were present in the perfusion buffer starting 15 min before the first bolus injection. CGP42112A-induced effects were also studied in combination with the α_1-adrenoceptor agonist phenylephrine, by injecting 1 mM phenylephrine alone or simultaneously with 0.1 μM CGP42112A.

Data analysis

CF and LVSP data were recorded and digitalized using WinDaq waveform recording software (Dataq Instruments, Akron, OH, U.S.A.). After a manual selection of the desired signals pre- and post-injection, data were analyzed using Matlab (Mathworks Inc., Natick, MA, U.S.A.). Six consecutive beats were selected for determination of CF and LVSP.

Data are given as mean $\pm$ s.e.m. and represent percentage change from baseline. Concentration–response curves were analyzed as described (DeLean *et al*., 1978), using Graph Pad Prism 3.01 (Graph Pad Software Inc., San Diego, CA, U.S.A.), to obtain pEC_{50} ($-^{10}\log EC_{50}$) and E_{max} values. The pEC_{50} values refer to the agonist concentration in the injection fluid and do not reflect the actual concentrations seen by the receptor. Statistical analysis between groups was by Student's *t*-test or one-way analysis of variance, followed by *post hoc* evaluation according to Dunnet. $P < 0.05$ was considered significant.

Results

Baseline hemodynamic values and effect of KH buffer injection

Baseline CF values were 2.7 ± 0.1 ml min^{-1} ($n = 50$), 2.7 ± 0.2 ml min^{-1} ($n = 5$) and 2.5 ± 0.2 ml min^{-1} ($n = 8$) in C57BL/6 mice, $AT_{1A}^{-/-}$ mice and $AT_{1A}^{+/+}$ wild-type control mice, respectively. Baseline LVSP values were 111 ± 3, 114 ± 8 and 99 ± 4 mmHg, respectively. KH buffer injections did not significantly affect these baseline parameters (Figures 1–4).

Studies in C57BL/6 mice

Ang II ($n=14$) concentration dependently decreased CF and LVSP, by maximally 41 ± 4 and $25\pm3\%$, respectively (pEC_{50}s 7.41 ± 0.12 and 7.65 ± 0.12; Figure 1). Ang II concentrations $>1\,\mu$M did not result in effects that were larger than those observed at $1\,\mu$M, in agreement with the concept of receptor desensitization (Abdellatif *et al*., 1991; Reagan *et al*., 1993; Iglesias *et al*., 2001). The Ang II effects were maximal within 10–20 and 20–30 s for CF and LVSP, respectively. Values returned to baseline after 0.5–1 min.

Irbesartan ($n=4$) abolished all Ang II-induced changes. In contrast, PD123319 ($n=6$) enhanced the effect of Ang II on CF (to $59\pm1\%$; $P<0.05$ vs control) and LVSP (to $44\pm2\%$; $P<0.05$ vs control), without altering its potency (pEC_{50}s 7.41 ± 0.12 and 7.49 ± 0.20, respectively). L-NAME ($n=4$) similarly enhanced ($P<0.05$) the effect of Ang II on CF (to $57\pm1\%$) and LVSP (to $35\pm2\%$), without altering its potency (pEC_{50}s 6.95 ± 0.34 and 7.22 ± 0.13, respectively; Figure 1). PD123319 ($n=4$) no longer enhanced the effect of Ang II on top of L-NAME, thereby indicating that its effect depends on NO.

Phenylephrine ($n=4$) decreased CF and LVSP (Figure 2, $P<0.05$). CGP42112A ($n=4$) did not diminish the constrictor and inotropic response to phenylephrine, nor did this AT_2 receptor agonist ($n=4$) exert constrictor or inotropic effects of its own (Figure 2). Bradykinin ($n=6$) increased CF by maximally $42\pm6\%$ and marginally affected LVSP (Figure 3).

Studies in $AT_{1A}^{-/-}$ mice

Ang II ($n=5$) did not affect CF or LVSP in $AT_{1A}^{-/-}$ mice, whereas the Ang II ($n=7$) response in $AT_{1A}^{+/+}$ wild-type control mice was indistinguishable from that in C57BL/6 mice (Figures 1, 4 and 5). Endothelin-1 (0.1 nM) decreased CF in both $AT_{1A}^{-/-}$ and $AT_{1A}^{+/+}$ wild-type control mice (Figure 5). The endothelin-1-induced decreases in CF and LVSP (47 ± 15 and $41\pm10\%$, respectively) were comparable to those induced by 1 mM phenylephrine (Figure 4).

Discussion

This study is the first to support the concept of AT_2 receptor-mediated vasodilation in the mouse coronary vascular bed. Evidence for such vasodilation was obtained indirectly, that is, as an enhanced constrictor response to Ang II in the presence of the AT_2 receptor antagonist PD123319. Data are in full agreement with previous studies on this matter in human (Batenburg *et al*., 2004), porcine (Zhang *et al*., 2003), rabbit (Pörsti *et al*., 1993) and rat (Schuijt *et al*., 2001) coronary arteries.

Vasodilation did not occur when exposing the mouse heart to Ang II in the presence of irbesartan (a condition allowing selective AT_2 receptor stimulation, which has been used successfully in previous studies; Munzenmaier & Greene, 1996; Widdop *et al*., 2002; Batenburg *et al*., 2004), nor during exposure of the heart to the AT_2 receptor agonist CGP42112A. This was not due to an inability to detect vasodilation, as bradykinin exerted its well-known vasodilator effects in our Langendorff setup. Furthermore, Ang II exerted no effect in hearts of $AT_{1A}^{-/-}$ mice, although these mice do express AT_2 receptors (Harada *et al*., 1998; Ryan *et al*., 2004). This demonstrates first that the AT_{1A} receptor is the receptor responsible for coronary vasoconstriction, in agreement with the observation that deletion of the AT_{1A} receptor (Ito *et al*., 1995), but not of the AT_{1B} receptor (Chen *et al*., 1997), virtually abolishes the *in vivo* vasoconstrictor response to Ang

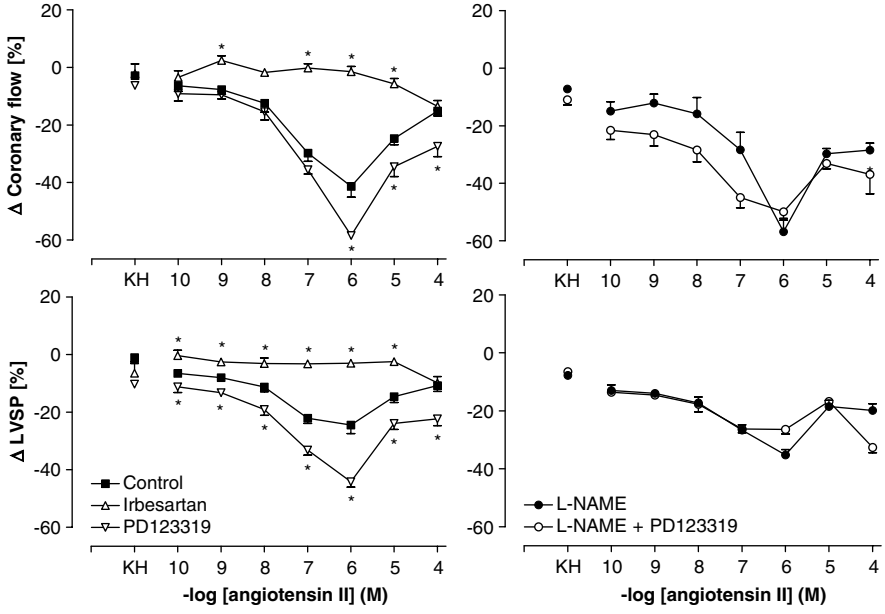

Figure 1 Left panels, effects of Ang II bolus injections (100 μl) on CF and LVSP in the mouse Langendorff heart in the absence (control; $n=14$) or presence of 1 μM irbesartan ($n=4$) or 1 μM PD123319 ($n=6$). Right panels, effects of Ang II bolus injections on CF and LVSP in the mouse Langendorff heart in the presence of 1 μM L-NAME with or without 1 μM PD123319 ($n=4$ for both conditions). The *x*-axis displays the Ang II concentration in the injection fluid. Data are mean$\pm$s.e.m. and represent percentage change from baseline. KH, bolus injection of Krebs–Henseleit buffer. *$P<0.05$ vs control.

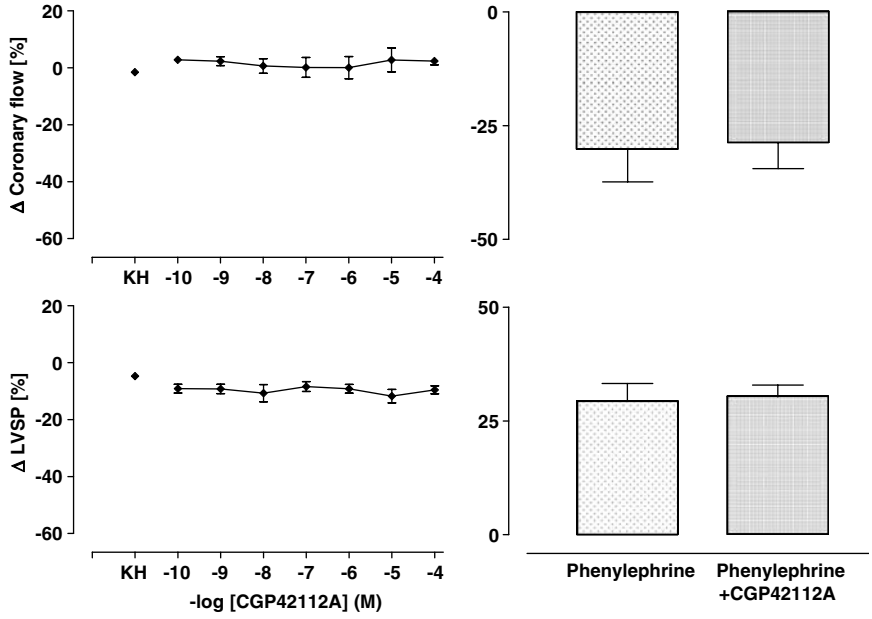

Figure 2 Left panels, effects of CGP42112A ($n=4$) bolus injections (100 μl) on CF and LVSP in C57BL/6 mice. The x-axis displays the concentration in the injection fluid. KH, bolus injection of Krebs–Henseleit buffer. Right panels, effects of a phenylephrine bolus injection (100 μl of a solution containing 1 mM phenylephrine), with or without 0.1 μM CGP42112A ($n=4$ for both conditions), on CF and LVSP in the mouse Langendorff heart. Data are mean $\pm$ s.e.m. and represent percentage change from baseline.

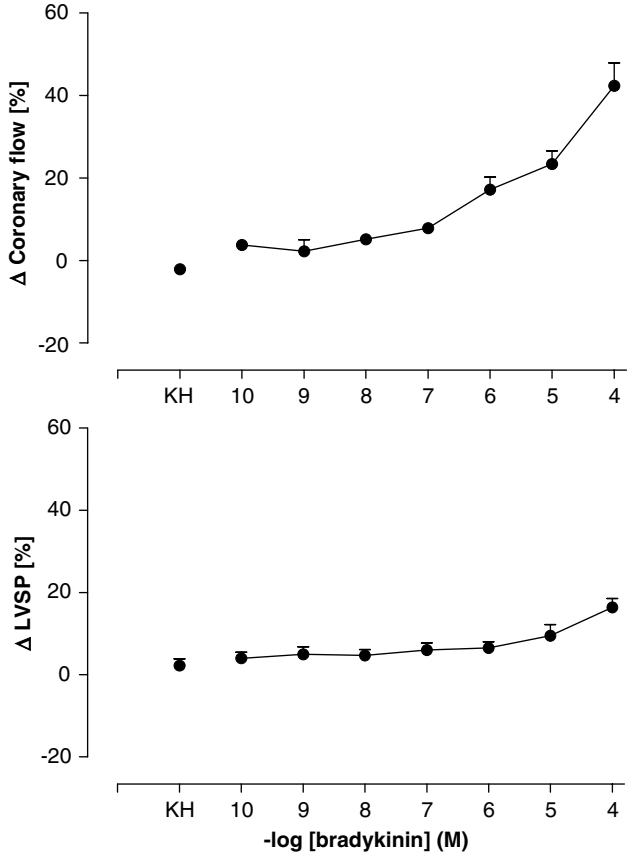

Figure 3 Effect of bradykinin bolus injections (100 μl) on CF and LVSP in the mouse Langendorff heart. Data are mean $\pm$ s.e.m. of six experiments and represent percentage change from baseline. KH, bolus injection of Krebs–Henseleit buffer.

II and reduces blood pressure. Secondly, it demonstrates that stimulation of AT_2 receptors in the absence of AT_{1A} receptors also does not result in vasodilation.

It has been suggested that AT_2 receptor-mediated vasodilation can only be observed in hypertensive (and not normotensive) animals (Li & Widdop, 2004). Thus, concomitant vasoconstriction might be a prerequisite to observe AT_2 receptor-mediated vasodilation. However, in contrast with this concept, CGP42112A did not affect the vasoconstrictor response to the α_1-adrenoceptor agonist phenylephrine in the mouse heart.

It appears therefore that AT_2 receptor-mediated effects depend on simultaneous AT_{1A} receptor activation, for instance because both receptors heterodimerize (AbdAlla *et al*., 2001), or because interaction occurs at the postreceptor level. Heterodimerization would require the simultaneous occurrence of both receptors in the same (smooth muscle) cell, and although this concept has been tested in smooth muscle cells of transgenic mice (Nakajima *et al*., 1995; Tsutsumi *et al*., 1999), most studies suggest that AT_2 receptors are restricted to endothelial cells (Stoll *et al*., 1995; Muller *et al*., 1998; Batenburg *et al*., 2004), whereas AT_1 receptors are mainly located on smooth muscle cells.

AT_2 receptor-mediated responses, in contrast with AT_1 receptor-mediated constriction, depend on both endothelial and smooth muscle cells, and involve a cascade starting with endothelial bradykinin type 2 receptor activation and subsequent NO synthesis, and finally resulting in guanylyl cyclase activation in smooth muscle cells (Tsutsumi *et al*., 1999; Hannan *et al*., 2003; Batenburg *et al*., 2005). In agreement with the important contribution of NO to the vasodilator effect of the AT_2 receptor, L-NAME enhanced the Ang II response to the same degree as PD123319, and

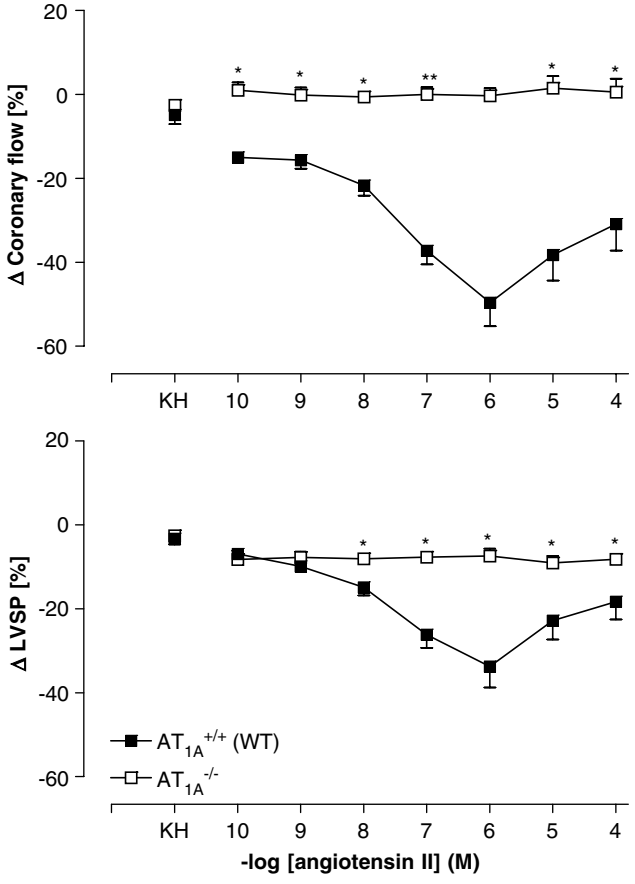

Figure 4 Effects of Ang II bolus injections on CF and LVSP in $AT_{1A}^{-/-}$ ($n=5$) and the corresponding $AT_{1A}^{+/+}$ (wild-type control; $n=7$) mice. The *x*-axis displays the concentration in the injection fluid. Data are mean ± s.e.m. and represent percentage change from baseline. KH, bolus injection of Krebs–Henseleit buffer. $^*P<0.05$ vs $AT_{1A}^{+/+}$.

PD123319 no longer enhanced the effect of Ang II in the presence of L-NAME.

The question then arises why direct, AT_2 receptor-mediated vasodilation could not be observed in the present study. First, although several groups have demonstrated such vasodilation in various species (including humans), both *in vitro* and *in vivo* (Endo *et al*., 1998; Dimitropoulou *et al*., 2001; Katada & Majima, 2002; Widdop *et al*., 2002; Batenburg *et al*., 2004), we are not aware of studies in mice showing Ang II-induced vasodilation. Thus, the simplest explanation is that mice differ from other species, in that their coronary AT_2 receptors are not limited to endothelial cells. Indeed, Utsunomiya *et al*. (2005) observed abundant AT_2 receptor protein immunoreactivity in the media of mouse coronary arteries. Second, Widdop *et al*. (2003) have suggested that the sensitivity of some experimental preparations is too low to observe AT_2 receptor-induced vasodilation. However, based on the robust (≈50%) PD123319-induced increase of the coronary constrictor response to Ang II, a considerable degree of vasodilation should have occurred in our preparation during selective AT_2 receptor stimulation, and such robust vasodilation was in fact present when exposing the heart to bradykinin, the putative mediator of the AT_2 receptor-mediated relaxation. Finally, application of Ang II *via* bolus injections differs greatly from the local production of Ang II in close proximity of AT_1 and AT_2 receptors (van Kats *et al*., 1998; Schuijt *et al*., 2002; Tom *et al*., 2003) that occurs *in vivo*, and thus, one further possibility is that arterial Ang II delivery is an inappropriate tool to observe AT_2 receptor-mediated vasorelaxation.

PD123319 enhanced the negative inotropic response to Ang II in the mouse heart. This suggests that AT_2 receptors counteract the AT_1 receptor-mediated negative inotropic effects in mouse cardiomyocytes. However, selective AT_2 receptor stimulation did not affect cardiac inotropy. Combined with the observation that AT_2 receptors do not occur in

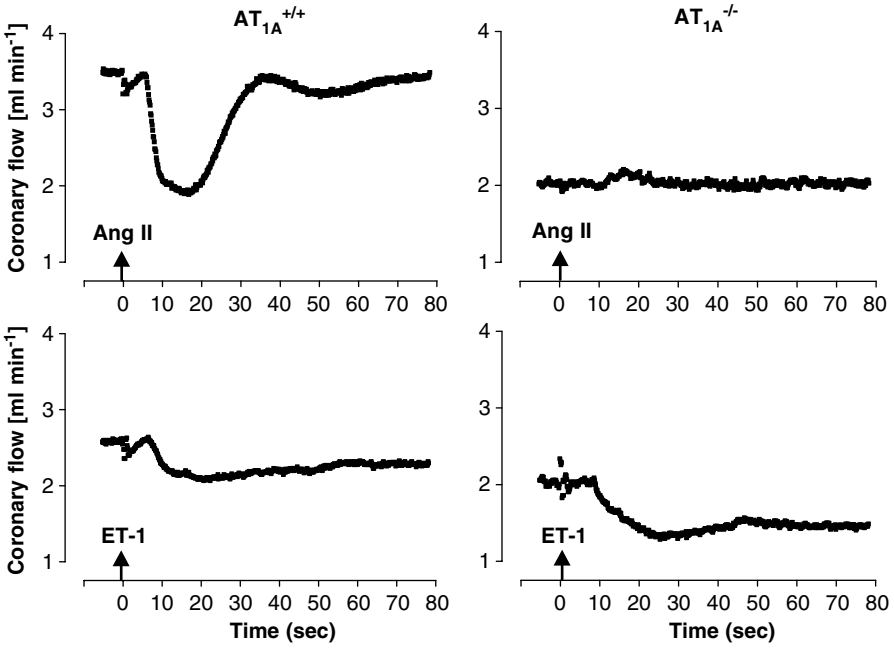

Figure 5 Representative tracings showing the effects of a bolus injection (100 μl; arrow) containing 1 μM Ang II or 0.1 nM endothelin-1 (ET-1) on CF in $AT_{1A}^{+/+}$ (wild-type control; left panels) and $AT_{1A}^{-/-}$ (right panels) mice.

cardiomyocytes (Utsunomiya *et al*., 2005), a more likely explanation is that the inotropic effects of Ang II in the mouse heart are a consequence of its effects on CF rather than the consequence of direct stimulation of AT_1 and/or AT_2 receptor on cardiomyocytes. This may be different under pathological conditions, for example, following myocardial infarction, when AT_2 receptors improve left ventricular systolic function (Yang *et al*., 2002).

In summary, our study provides evidence for opposite effects of AT_{1A} and AT_2 receptors in the coronary vascular bed of normotensive mice. AT_2 receptor-mediated effects depended on NO and occurred only in conjunction with AT_{1A} receptor activation. The latter observation suggests that AT_{1A} and AT_2 receptors display an interaction, either directly (due to receptor heterodimerization) or at the postreceptor level. Such interaction might be of particular importance under conditions where the RAS is stimulated, for example, during sodium depletion or in subjects with renovascular hypertension. A similar functional interaction has been described between the Ang-(1–7) receptor Mas and AT_1 and AT_2 receptors (Castro *et al*., 2005). Future investigations should elucidate the exact site of AT_{1A}–AT_2 receptor interaction and whether the interaction is altered under pathological conditions.

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