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## No involvement of the AT<sub>2</sub>-receptor in angiotensin II-enhanced sympathetic transmission *in vitro*

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### Abstract

Angiotensin II (Ang II) enhances sympathetic neurotransmission via AT<sub>1</sub>-receptors located on sympathetic nerve terminals. We recently demonstrated that inhibition of Ang II-mediated facilitation in the pithed rat by irbesartan resulted in a U-shaped dose response curve, which was not observed when PD 123319, at a concentration that selectively blocks the AT<sub>2</sub>-receptor, was co-administered. Hence, the irbesartan-mediated 'upstroke' might be explained by the involvement of the AT<sub>2</sub>-receptor after AT<sub>1</sub> blockade with high-dose irbesartan. In the present study, we further investigated the possible role of the AT<sub>2</sub>-receptor in Ang II-mediated facilitation *in vitro*.

We studied the effect of the AT<sub>2</sub>-receptor antagonist PD 123319 (10 nM) on Ang II-enhanced sympathetic outflow evoked by electrical field stimulation (EFS) in the rat isolated inferior vena cava. Additionally, we investigated the effect of the AT<sub>1</sub>-receptor blocker irbesartan (0.1 nM–1 μM) on the sequelae of Ang II-enhanced, EFS-evoked sympathetic nerve traffic in the presence or absence of PD 123319 (10 nM).

PD 123319 did not influence Ang II-enhanced sympathetic outflow. Irbesartan dose-dependently attenuate Ang II-augmented transmitter release (pIC<sub>50</sub> 7.99±0.03), whereas no U-shaped concentration-response relationship for irbesartan was observed. Co-administration of PD 123319 with irbesartan proved unable to influence Ang II-mediated facilitation differently compared with irbesartan alone. The experimental observations indicate that the AT<sub>2</sub>-receptor is not involved in Ang II-mediated enhancement of sympathetic nerve traffic in the present *in vitro* study.

### Introduction

Angiotensin II (Ang II) has been shown to interact with the sympathetic nervous system (SNS) at several levels. These interactions involve the enhancement of noradrenergic neurotransmission at central nervous structures, the sympathetic ganglia and the adrenals, as well as at peripheral sympathetic nerve terminals.<sup>1,2</sup>

The receptors through which Ang II exerts its effects are subclassified into AT<sub>1</sub>- and AT<sub>2</sub>-receptors. The AT<sub>1</sub> subtype is sensitive to the reference compound losartan whereas the AT<sub>2</sub>-subtype is sensitive to PD 123177 and to low concentrations of the related agent PD 123319.<sup>3,6</sup>

Although it has been claimed that the majority

of effects elicited by Ang II are mediated through the AT<sub>1</sub>-receptor subtype,<sup>7</sup> the physiological role of the AT<sub>2</sub>-receptor is the focus of a great deal of recent attention. Cardiovascular remodelling, vasodilatation, apoptosis, foetal development and anti-thrombotic activity are some of the effects presumed to be mediated via the AT<sub>2</sub>-receptor.<sup>8-10</sup>

The AT<sub>1</sub>-receptor is known to mediate the facilitatory actions of Ang II on noradrenergic neurotransmission.<sup>11-13</sup> Recently, however, several studies have suggested that, besides AT<sub>1</sub>-involvement, the AT<sub>2</sub>-receptor may be associated with Ang II-enhanced sympathetic nerve traffic. In conscious rats, administration of Ang II into the cerebral ventricles resulted in significant pressor effects, which were associated with a marked vasoconstriction at the level of the mesenteric and hindquarter vascular beds. Both PD 123319 and EXP-3174, administered into the cerebral ventricles, abolished the cardiovascular response to central Ang II infusion, thus indicating the involvement of the AT<sub>2</sub>-receptor.<sup>14</sup> In addition, PD 123319 (0.01 and 0.1 μM) abolished the synergistic interaction between losartan (10 nM) and Ang II, in terms of stimulation-induced noradrenaline efflux as well as nerve stimulated vasoconstriction in the rat caudal artery.<sup>15</sup> This phenomenon might be explained by an unmasking of a latent population of AT<sub>2</sub>-receptors that subserves further facilitation.

In accordance with these observations, we recently demonstrated that inhibition of Ang II-mediated facilitation in the pithed rat by irbesartan (1–60 mg/kg) resulted in a U-shaped dose response curve.<sup>16</sup> The highest dose of irbesartan caused less than maximal sympatho-inhibition. This U-shaped dose-response relationship was not observed when PD 123319 (5 mg/kg+50 μg/kg/minute) was co-administered. Hence, the irbesartan-mediated 'upstroke' may be explained by the involvement of the AT<sub>2</sub>-receptor after AT<sub>1</sub>-blockade with high-dose irbesartan.

It was the objective of the present study to further investigate the possible role of the AT<sub>2</sub>-receptor in Ang II-mediated facilitation *in vitro*. We applied the noradrenaline spillover technique to study the influence of selective AT<sub>1</sub>- and AT<sub>2</sub>-receptor blockade on the exocytotic release of neurotransmitter. This straightforward model was shown to be suitable to investigate the interactions between the renin angiotensin system (RAS) and the peripheral SNS.<sup>17</sup>

Accordingly, we studied the effects of the

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selective AT<sub>2</sub>-receptor antagonist, PD 123319, on Ang II-enhanced sympathetic outflow evoked by electrical field stimulation (EFS) in the isolated inferior vena cava of the rat. Additionally, we investigated the inhibitory effect of irbesartan on the sequelae of Ang II-enhanced, EFS-evoked sympathetic nerve traffic in the presence or absence of PD 123319. By selectively excluding the AT<sub>1</sub>-receptor (irbesartan), we intended to address or unmask a possible latent population of AT<sub>2</sub>-receptors, as we previously described for the pithed rat preparation. Subsequently, we antagonised this receptor population by applying PD 123319. Furthermore, we determined the sympatholytic potency of irbesartan in the present model.

### Materials and methods

The experimental protocol was approved by the Committee on Animal Experiments of the Academic Medical Centre, Amsterdam. Male Wistar rats, weighing 240–260 g, were used.

The rats were stunned and decapitated. The thoracic cavity was opened and the inferior vena cava was dissected free from its connective tissue and transferred to a petri plate containing physiological salt solution (PSS), gassed with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> at room temperature.

#### Rat isolated vena cava inferior preparations

The proximal and distal ends of the vena cava were ligated with fine silk threads and the preparation transferred to an organ bath.

The PSS was composed as follows (mmol/L): NaCl 118, Na<sub>2</sub>HPO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, KCl 4.7, CaCl<sub>2</sub> 1.6, MgSO<sub>4</sub> 1.2, and glucose 11.0. Ascorbic acid (0.3 mmol/L) and Na<sub>2</sub>EDTA (0.03 mmol/L) were added to prevent the oxidation of noradrenaline.

#### Radiolabelling of noradrenergic transmitter stores

In order to label their noradrenergic vesicles, the isolated veins were incubated for 45 minutes in 2.0 ml of PSS containing 0.1 mmol/l 1-[7,8-<sup>3</sup>H]-noradrenaline (specific activity 28.8 to 52.0 Ci/mmol) in a 5 ml glass-jacketed organ bath. The medium was continuously bubbled with carbogen and maintained at a temperature of 37°C.

After the incubation period, the isolated veins were washed with [<sup>3</sup>H]-noradrenaline-free PSS (10-x 2 ml and 4-x 5 ml) to remove superficially bound, non-neuronal radioactivity before the experimental procedures were started. After the wash procedure, the veins were mounted vertically between platinum wire electrodes (2 cm) placed along either side of the preparations in a 25 ml organ bath and subjected to a tension of 0.5 g.

The organ bath contained 20.0 ml PSS. Desipramine (0.6 μmol/L) and corticosterone (40 μmol/L) were added in order to rule out uptake-1 and uptake-2 of [<sup>3</sup>H]-noradrenaline, respectively. Yohimbine (1 μmol/L) was added to the PSS to rule out any α<sub>2</sub>-adrenergic auto-inhibitory effects on [<sup>3</sup>H]-noradrenaline release. The veins were equilibrated for a total period of 48 minutes. After an initial period of 18 minutes, the preparations

were subjected to a 2-minute period of EFS with a train of 3 ms rectangular bipolar wave pulses of 150 mA, at a frequency of 2 Hz (S<sub>1</sub>) (Danish Myo Technology Current Stimulator, model CS 200). This 'priming' stimulation has been proven to increase the reliability and stability of the subsequent basal and EFS-induced [<sup>3</sup>H]-noradrenaline spillover.

#### Stimulation of intrinsic sympathetic nerves

After the equilibration period, veins were subjected to two additional periods of EFS (trains of 2 minutes, 3 ms, 150 mA, 2 Hz). The second period of stimulation (S<sub>2</sub>) was applied directly after the equilibration period of 48 minutes, and the tritium outflow thus evoked was taken as the control value. Subsequently, a third period (S<sub>3</sub>) was applied 24 minutes after S<sub>2</sub>. The ratio between S<sub>3</sub> and S<sub>2</sub> was calculated to quantify the influence exerted by the drugs to be investigated.

#### Measurement of tritium outflow

Samples of 0.5 ml each were repeatedly taken from the organ bath, starting at 36 minutes after washout. The actual tritium outflow could be obtained by calculating the incremental accumulation in each sample. We corrected for the reduced volume, which decreased stepwise by the repeated drawing of samples.

The mean basal tritium efflux/minute preceding the stimulation periods S<sub>2</sub> and S<sub>3</sub> was determined as the mean outflow/minute of tritium in two 6-minute samples prior to each period of stimulation. For S<sub>2</sub>, we subtracted the radioactivity (CPM) measured in the sample taken at t=36 minutes from that of t=42 minutes and the radioactivity measured in the sample taken at t=42 minutes from that of the sample at t=48 minutes. Accordingly, we could determine the outflow/minute of radioactivity during two 6-minute time intervals prior to S<sub>2</sub>. Basal outflow was determined by averaging these values. An equivalent procedure was applied for S<sub>3</sub>.

The release/minute evoked by EFS (S<sub>2</sub> and S<sub>3</sub>, 2-minute samples) was calculated by subtracting the corresponding mean basal efflux/minute from the apparent EFS-evoked efflux/minute.

At the end of the experiment, the residual radioactivity of the tissue was measured. By adding the total released tritium to this value, the initial content of tritium was calculated. The effect of EFS on the release could then be expressed as a fraction of the total tissue content present at the moment of stimulation, the 'fractional release' of radioactivity (FR<sub>2</sub> and FR<sub>3</sub>). Accordingly, the effects of pharmacological interventions are expressed as the ratio FR<sub>3</sub>/FR<sub>2</sub>.

#### Detection of tritium in the samples and tissue

After the experiment, the tissues were kept overnight in 2 ml of 0.5 M quarternary ammonium hydroxide dissolved in toluene (Soluene, Packard). Radioactivity was measured by liquid scintillation counting (Tri Carb 2900TR, Packard) in 20 ml

**Table 1** Absolute values of radioactivity (DPM), reflecting [<sup>3</sup>H]-noradrenaline outflow, by rat inferior vena cava preparations in control experiments. Subsequently are shown: basal outflow per sample prior to S<sub>2</sub>, total tritium content at the start of the experiments, FR<sub>2</sub> values (absolute and relative to the amount contained by the venae at S<sub>2</sub>) and the ratio of the relative radioactivity evoked by S<sub>3</sub> and S<sub>2</sub>

Basal outflow (DPM)	Total tritium content (DPM)	Absolute value FR <sub>2</sub> (DPM)	FR <sub>2</sub> (% of total at S <sub>2</sub> )	FR <sub>3</sub> /FR <sub>2</sub>	n
3.29±0.66 × 10 <sup>3</sup>	4.55±0.59 × 10 <sup>5</sup>	1.15±0.14 × 10 <sup>4</sup>	2.61±0.17	0.70±0.02	7

aliquots (with either samples or tissue) after addition of 5 ml of the scintillation fluid (Ultima Gold, Packard). Corrections for counting efficiency were made by external automatic standardisation.

### Experimental design

Three different experiments were performed:

1. To investigate the influence of Ang II on EFS-evoked noradrenaline release, Ang II was added to the medium 150 seconds prior to S<sub>3</sub>. Three different concentrations were studied; 1, 10 and 100 nM, respectively. To express the effect of Ang II the ratio FR<sub>3</sub>/FR<sub>2</sub> was used.
2. In another series of experiments, we studied the influence of the selective AT<sub>1</sub>-antagonist, irbesartan (1 nM–1 μM), and the selective AT<sub>2</sub>-blocker, PD 123319 (10 nM), on Ang II-enhanced EFS-evoked sympathetic outflow. Either irbesartan, PD 123319 (10 nM) or vehicle was added to the medium 20 minutes before S<sub>3</sub>. Ang II (10 nM) was added 150 seconds prior to S<sub>3</sub>. To characterise the sympatho-inhibitory effects of irbesartan and PD 123319, the ratio FR<sub>3</sub>/FR<sub>2</sub> was used. PD 123319 (10 nM) is known to be effective in blocking the AT<sub>2</sub>-receptor.
3. In a third series of experiments, we investigated whether AT<sub>1</sub>-inhibition by irbesartan (1 and 10 nM), combined with AT<sub>2</sub>-blockade by PD 123319 (10 nM), could influence Ang II-mediated facilitation differently when compared with inhibition by irbesartan alone, thus demonstrating the presence and function of the AT<sub>2</sub>-receptor. Irbesartan (1 or 10 nM) and PD 123319 (10 nM), or vehicle was added to the medium 20 minutes before S<sub>3</sub>. Ang II (10 nM) was added 150 seconds prior to S<sub>3</sub>. To characterise the effects of irbesartan and PD 123319, the ratio FR<sub>3</sub>/FR<sub>2</sub> was used.

### Drugs and chemicals

Irbesartan (Sanofi, Amilly, France) was dissolved in 1M NaOH. The pH of the solution was adjusted to 7.5 using 1M HCl. Ang II (Bachem, Bubendorf, Switzerland, synthetic human sequence) and PD 123319 (Parke Davis, Ann Arbor, USA) were dissolved in distilled water. Stock solutions of Ang II (1.10<sup>3</sup>, 1.10<sup>4</sup>) were stored in 50 μl aliquots at -20°C.

Desipramine HCl (Sigma, St Louis, USA) and yohimbine HCl (Sigma, St Louis, USA) were dissolved in distilled water. Hydrocortisone Hydrogensuccinate (Bufa, Uitgeest, Holland) was dissolved in DMSO. Stock solutions of desipramine

(6.10<sup>-4</sup>), yohimbine (1.10<sup>-3</sup>) and corticosterone (4.10<sup>-2</sup>) were further diluted with PSS. Tritiated levo-[7,8-<sup>3</sup>H]-noradrenaline (Amersham Pharmacia Biotech, Little Chalfont, England) had a specific radioactivity of 28.8–52.0 Ci/mmol and a radioactive concentration of 1.0 mCi/ml. Soluene and Ultima Gold solutions were obtained from Packard Bioscience (Groningen, Netherlands).

### Statistical analysis

All data are expressed as mean±S.E.M. Student's *t*-test (two-tailed unpaired) was used to evaluate the statistical significance of differences between means of control and treatment groups. ANOVA, followed by Dunnett's test, was performed for multiple comparisons with a control group. Differences at *p*<0.05 were considered statistically significant.

In order to determine the sympatho-inhibitory potency of irbesartan, we used a computer programme (GraphPad Prism, GraphPad, San Diego, USA). The curve was fitted to log concentration-effect data. The underlying equation is  $E = E_{\max} * A^p / (A^p + IC_{50}^p)^{-1}$ . In this equation, *E* is the response obtained at a given concentration *A*, *E*<sub>max</sub> is the maximally attainable response, *IC*<sub>50</sub> the concentration antagonist for the half maximal effect, \* denotes 'multiplied by', and the exponent *p* describes the slope of the relationship (Hill-coefficient). The curve was fitted to averaged concentration-effect data.

### Results

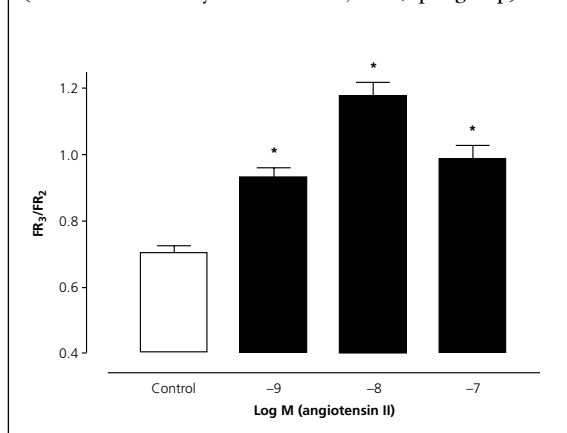
#### Basal parameters

In Table 1, the data concerning the basal outflow samples (DPM), the absolute fractional release FR<sub>2</sub> (DPM), the total tritium amount contained by the organ at the start of the experiments (DPM) and the fractional release FR<sub>2</sub> (as % total tritium) and the relative response to S<sub>3</sub> compared to S<sub>2</sub> (FR<sub>3</sub>/FR<sub>2</sub>) are summarised for the control experiments.

#### Experiment 1: angiotensin II and EFS-evoked tritium outflow

Ang II (1 nM–0.1 μM) did not influence the basal tritium efflux (data not shown). In contrast, Ang II caused a concentration-dependent increase in EFS-evoked noradrenaline spillover. All concentrations applied caused a significant enhancement of the spillover. The maximal facilitation, by Ang II (10 nM), amounted to 66.9% (FR<sub>3</sub>/FR<sub>2</sub> 1.18±0.04, *n*=9). Ang II (0.1 μM) evoked less than maximal enhancement of EFS-evoked sympathetic outflow, resulting in a 'bell-shaped' curve (Figure 1).

**Figure 1** Enhancing effect of angiotensin II (Ang II) (1 nM–0.1  $\mu$ M) on the EFS-evoked [ $^3$ H]-noradrenaline outflow from isolated rat vena cava inferior preparations. The preparations were stimulated at 24-minute intervals. Ang II was added to the organ bath 150 seconds before  $S_3$ . The ratio between fractional releases evoked by  $S_3$  (FR $_3$ ) and  $S_2$  (FR $_2$ ) is shown on the ordinate, for controls and Ang II, respectively. Columns represent means $\pm$ SEM.  $\square$  = control;  $\blacksquare$  = Ang II; \* $p$ <0.05 compared with control (ANOVA followed by Dunnett's test,  $n$ =6–9 per group)



### Experiment 2: irbesartan and PD 123319 and angiotensin II-facilitated [ $^3$ H]-noradrenaline outflow

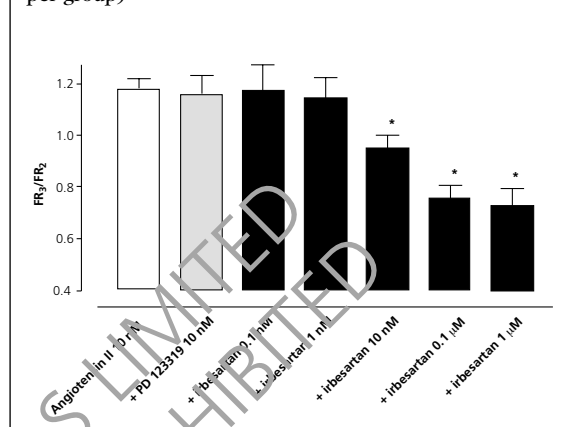
The highest concentrations of irbesartan (1  $\mu$ M) and PD 123319 (10 nM) neither influenced the basal efflux of tritium (data not shown) nor the EFS-evoked tritium spillover (FR $_3$ /FR $_2$  0.71 $\pm$ 0.12 and 0.71 $\pm$ 0.14, respectively) ( $n$ =4,  $p$ >0.05).

Moreover, PD 123319 (10 nM) did not influence the Ang II-facilitated (10 nM) EFS-evoked noradrenaline spillover (FR $_3$ /FR $_2$  1.16 $\pm$ 0.07) (Figure 2). Irbesartan, however, dose-dependently attenuated the subsequent Ang II-mediated (10 nM) enhancement of EFS-evoked sympathetic outflow. After exposure to the two lowest concentrations of irbesartan (0.1 and 1 nM), the noradrenaline spillover did not significantly differ from the EFS-evoked spillover in the presence of Ang II (10 nM) alone. Conversely, at the higher three concentrations used (10 nM–1  $\mu$ M) the AT $_1$ -antagonist significantly inhibited the Ang II-mediated responses ( $p$ <0.05). The IC $_{50}$  value, which is the concentration of irbesartan that causes 50% reduction of the enhancement of EFS-evoked spillover by Ang II (10 nM), amounted to -7.99 $\pm$ 0.03 (expressed as log M).

### Experiment 3: irbesartan combined with PD 123319 and angiotensin II-facilitated tritium outflow

AT $_1$ -inhibition by irbesartan (1 and 10 nM) combined with AT $_2$ -blockade by PD 123319 (10 nM) influenced Ang II-mediated facilitation. As in the experiment with irbesartan alone, irbesartan 10 nM (in combination with PD 123319 10 nM) significantly reduced the Ang II-enhanced noradrenaline spillover. However, when compared with the inhibitory effect of irbesartan alone, the combination of PD 123319 (10 nM) and either concentra-

**Figure 2** Inhibitory effect of irbesartan (0.1 nM–1  $\mu$ M) and PD 123319 (10 nM) on the facilitation by angiotensin II (Ang II) (10 nM) of EFS-evoked [ $^3$ H]-noradrenaline outflow from rat isolated vena cava inferior. The veins were stimulated at 24-minute intervals. Ang II (10 nM) in the presence or absence of irbesartan or PD 123319 was added to the organ bath 150 seconds prior to  $S_3$ . The antagonist were added 20 minutes before  $S_3$ . The ratio between fractional releases evoked by  $S_3$  (FR $_3$ ) and  $S_2$  (FR $_2$ ) is shown on the ordinate, the concentrations of the receptor antagonists on the abscissa.  $\square$  = Ang II (10 nM);  $\square$  = Ang II + PD 123319;  $\blacksquare$  = Ang II + irbesartan. Columns represent the mean $\pm$ SEM. \* $p$ <0.05 compared with the Ang II group (ANOVA followed by Dunnett's test,  $n$ =5–9 per group)



tion of irbesartan (1 and 10 nM) inhibited Ang II-mediated facilitation to the same extent (Figure 3).

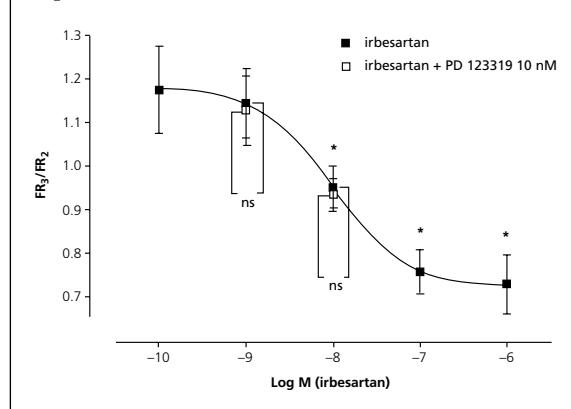
### Discussion

We deliberately compared the sympatho-inhibitory effects of AT $_1$ -blockade by irbesartan alone, and combined AT $_1$ /AT $_2$ -blockade by irbesartan plus PD 123319, on Ang II-augmented sympathetic nerve traffic. We observed no difference between the two approaches. Accordingly, we conclude that the AT $_2$ -receptor is not involved in prejunctional facilitation in the present *in vitro* investigation. The inability to demonstrate a U-shaped concentration-effect relationship for irbesartan supports this conclusion.

Previously, we demonstrated, in the pithed rat model, that the increase in diastolic blood pressure (DBP) to electrical stimulation of the thoracolumbar spinal cord could be dose-dependently inhibited by selective AT $_1$ -receptor blockade.<sup>16,18</sup> However, the highest dose of irbesartan (60 mg/kg) caused less than maximal attenuation of the rise in DBP. We hypothesised that unmasking of a latent population of AT $_2$ -receptors, that mediates further facilitation, might explain our observations. Indeed, the selective AT $_2$ -blocker, PD 123319 (5 mg/kg+50  $\mu$ g/kg/minute), could abolish the upstroke when co-administered with irbesartan.

In the present study, as to be expected, Ang II (1 nM–0.1  $\mu$ M) caused a concentration-dependent enhancement of EFS-evoked sympathetic nerve traffic, with a maximum of approximately 67% (at 10 nM) (see Figure 1). Previous studies reported similar magnitudes. In numerous vascular and cardiac tissues, Ang II-enhanced sympathetic

**Figure 3** Inhibitory effect of irbesartan (1 nM and 10 nM) combined with PD 123319 (10 nM) □, compared to irbesartan alone ■, on the facilitation by angiotensin II (Ang II) (10 nM) of EFS-evoked [<sup>3</sup>H]-noradrenaline outflow from rat vena cava inferior. The veins were stimulated at 24-minute intervals. Ang II (10 nM) in the presence of irbesartan or the combination irbesartan/PD 123319 was added to the organ bath 150 seconds prior to S<sub>3</sub>. The antagonists were added 20 minutes before S<sub>3</sub>. The ratio between fractional releases evoked by S<sub>3</sub> (FR<sub>3</sub>) and S<sub>2</sub> (FR<sub>2</sub>) is shown on the ordinate, the concentrations of the receptor antagonists (log M) on the abscissa. Data are presented as means±SEM. \*p<0.05 compared with the Ang II/irbesartan 0.1 nM group (ANOVA followed by Dunnett's test, n=5-9 per group). To evaluate statistical significance between irbesartan and the combination irbesartan/PD 123319 groups Student's *t*-test (two-tailed, unpaired) was used



transmission varied from approximately 60% to 90%.<sup>19,21</sup> Additionally, we observed a 'bell-shaped' concentration-effect relationship, as was shown previously.<sup>15,19,21</sup> This rather curious phenomenon might be explained by the concomitant release of anti-facilitatory prostaglandins.<sup>22</sup>

PD 123319, at a concentration known to block the AT<sub>2</sub>-receptor (10 nM),<sup>5</sup> did not influence the augmentation induced by Ang II (Figure 2). Therefore, it appears that the AT<sub>2</sub>-receptor does not play a significant role in Ang II-mediated facilitation, as was previously reported.<sup>23,24</sup>

Conversely, irbesartan dose-dependently attenuated Ang II-enhanced, EFS-evoked sympathetic transmission to baseline levels (see Figure 2), thus confirming the general view that Ang II-facilitated sympathetic nerve traffic is mediated through the AT<sub>1</sub>-receptor subtype.<sup>11,15,25</sup>

The sympatholytic potency (pIC<sub>50</sub>) of irbesartan amounted to 7.99±0.03. We previously reported a pIC<sub>50</sub> value of irbesartan of 9.20±0.03 in the same model using rabbit thoracic aortic ring preparations.<sup>15</sup> This discrepancy might be explained by species differences (rat versus rabbit). However, the Ang II concentration used in the present study (10 nM) differed 10-fold (1 nM) from that used previously, which is more likely to be responsible for the discrepancy.

Interestingly, we did not observe a U-shaped dose-response relationship for irbesartan, in contrast to our previous findings with high-dose irbesartan in the pithed rat model.<sup>16,18</sup> However, several *in vitro* studies, by us and others, investigating the

pharmacological properties of AT<sub>1</sub>-receptor antagonists concerning Ang II-enhanced transmission, failed to show an U-shaped concentration-response relationship.<sup>12,13,20</sup>

Several explanations are possible for these contrasting findings. First, the prejunctional AT<sub>2</sub>-receptor might be (functionally) absent *in vitro*, possibly by down-regulation, as has been described for murine neuronal cells.<sup>26</sup> Similarly, in the pithed rat, postjunctional α<sub>2</sub>-adrenoceptor-mediated vasoconstriction can be demonstrated to occur.<sup>27</sup> By contrast, functional α<sub>2</sub>-adrenoceptors cannot be demonstrated *in vitro* in various models.<sup>28,29</sup> Similarly, the same concept of *in vivo/in vitro* differences may explain the lack of 'upstroke' observed in the current study. Conversely, at the postjunctional site, the AT<sub>2</sub>-receptor appears to remain present, since Ang II (with AT<sub>1</sub>-blockade) induced a concentration-dependent relaxation in rat isolated mesenteric arteries, which could be inhibited by PD 123319.<sup>30</sup> Secondly, the maximal concentration of irbesartan (1 μM) used in the present study may not be sufficient to unmask a latent population of AT<sub>2</sub>-receptors and hence no upstroke can be observed. This explanation, however, is very unlikely regarding the plateau that was reached using the two highest concentrations of irbesartan.

As suggested by several authors, the beneficial effects of AT<sub>1</sub>-receptor blocker (ARB) therapy in hypertension and heart failure may, at least partly, be explained by Ang II-mediated stimulation of the unopposed AT<sub>2</sub>-receptor.<sup>31-33</sup> We therefore attempted to demonstrate a role for the putative AT<sub>2</sub>-receptor in Ang II-mediated facilitation by partly blocking the prejunctional AT<sub>1</sub>-receptor. When irbesartan (1 and 10 nM) was administered together with PD 123319 (10 nM) (combined AT<sub>1</sub> and AT<sub>2</sub> blockade), the sympatholytic effects of irbesartan were not different from those of irbesartan-mediated inhibition alone (see Figure 3). Consequently, as suggested earlier, it appears that the AT<sub>2</sub>-receptor is not present at the level of the sympathetic nerve terminals *in vitro*.

In conclusion, the facilitating effect of Ang II on EFS-evoked sympathetic nerve traffic is mediated exclusively by prejunctional AT<sub>1</sub>-receptors. The Ang II-mediated enhancement could be concentration-dependently antagonised (pIC<sub>50</sub> 7.99±0.03) by the selective AT<sub>1</sub>-receptor antagonist, irbesartan. The selective AT<sub>2</sub>-receptor antagonist, PD 123319, either alone or in combination with partial AT<sub>1</sub>-blockade with irbesartan, proved unable to influence Ang II-mediated facilitation. Therefore, we conclude that the AT<sub>2</sub>-receptor is not involved in Ang II-mediated enhancement of sympathetic nerve traffic *in vitro*.

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