Prejunctional and postjunctional inhibitory actions of eprosartan and candesartan in the isolated rabbit mesenteric artery
**Chapter 5**

**Introduction**

In numerous studies, the interaction of angiotensin II (Ang II) with the sympathetic nervous system (SNS) at several sites such as the central nervous system, the sympathetic ganglia, the adrenal medulla and at post-ganglionic nerve terminals have been demonstrated [1-4]. Possible mechanisms by which Ang II may enhance sympathetic neurotransmission include an increase of stimulation-induced (S-I) release of noradrenaline (NA), an inhibition of its uptake and an increased responsiveness of vascular smooth muscle to NA. The receptors through which Ang II exerts its effects are divided into the AT\(_1\) (sensitive to the reference compound losartan) and AT\(_2\) (sensitive to PD123177 and PD 123319) subpopulations, respectively [5-7]. Selective AT\(_1\)-antagonists can inhibit the enhancing effect of Ang II on sympathetic neurotransmission whereas no effect of AT\(_2\)-blockers is observed [8,9]. Several new, non-peptidergic AT\(_1\)-selective receptor blockers are now available. A few studies have recently been published in which the potency of these drugs with respect to the attenuation of the facilitation by Ang II of sympathetic neurotransmission was compared [10-14]. From the studies cited above, it is clear that all AT\(_1\)-antagonists can attenuate this facilitation. However, differences in potency have been described between the various AT\(_1\)-antagonists regarding inhibition of the presynaptic site. It now appears to be of interest to compare the pre- and postsynaptic activities of the various AT\(_1\)-blockers under carefully standardized conditions.

We recently described that in the pithed rat model, in which we compared four different AT\(_1\)-antagonists (valsartan, candesartan, eprosartan and embusartan), the order of potency of these compounds regarding inhibition of the presynaptic site, differs from the order of potency regarding inhibition of direct vasoconstrictor effects of Ang II [15]. Moreover, we found that for eprosartan, sympatho-inhibitory concentrations on the one hand, and concentrations needed for postsynaptic blockade on the other, differed far less than for candesartan. These findings may be explained by differences in receptor subtype between the pre- and postsynaptic AT\(_1\)-receptors.

The present study was designed to verify these findings in an in vitro set-up. Accordingly, it was the objective of the present study to (1) study the effect of Ang II on sympathetic neuroeffector transmission in the isolated rabbit mesenteric artery; (2) to compare the potency of the selective AT\(_1\)-receptor antagonists eprosartan and candesartan regarding inhibition of pre- and postjunctional AT\(_1\)-receptors; (3) to explore whether AT\(_1\)-receptors play a role in the facilitating effects of Ang II on sympathetic neurotransmission.
Prejunctional and postjunctional AT₁-blockade by eprosartan and candesartan

To investigate blockade of pre-synaptic AT₁-receptors and AT₂-receptors we studied the effect of the AT₁-antagonists eprosartan and candesartan and the AT₂-antagonist PD123319 on Ang II induced facilitation of stimulation-induced vasoconstriction. To investigate the role of postsynaptic AT₁-receptor blockade we investigated the effect of the AT₁-antagonists on concentration response curves elicited by exogenous Ang II. In addition, the effect of AT₁-blockade on post-synaptic α-adrenoceptor mediated responses was investigated by means of exogenously administered noradrenaline (NA). In the present study, multiple concentrations of each AT₁-antagonist were used, enabling us to compare both sympatho-inhibitory and vasopressor-inhibiting potencies on the basis of concentration-response relationships.
Methods

The experimental protocol was approved by the committee on Animal Experiments of the Academic Medical Center Amsterdam. New Zealand White rabbits of either sex weighing 2000 – 3400 g were used. The rabbits were anaesthetized with Hypnorm® (fentanyl/fluanison) 2.5 mg/kg i.m. Subsequently, heparin 875 IE/kg i.v. was injected and rabbits were killed with Nembutal® (pentobarbital) 60 mg/kg i.v. The mesentery and intestine were removed and placed in physiological salt solution (PSS) which was gassed with a mixture of 95% O₂ and 5% CO₂ at room temperature. Four segments of mesenteric artery with a length of ~2 mm each were dissected and a stainless steel wire with a diameter of 40 μm was inserted into the lumen of each vessel. The vessels were then transferred into the organ baths of an isometric wire myograph. The organ bath contained PSS of the following composition (mM): NaCl 118.5, KCl 4.7, MgCl₂ 1.2, CaCl₂ 2.5, KH₂PO₄ 1.2, NaHCO₃ 25, glucose 5.5, EDTA 0.024. Ascorbic acid (100 mg/l) was added to prevent oxidation of noradrenaline. Propranolol (1 μM), yohimbine (1 μM) and N°-Nitro-L-Arginine (L-NNA) (0.1 mM) were added in order to exclude the β-adrenergic and α₁-adrenergic effects of noradrenaline and the influence of endothelium-derived NO, respectively. The preparations were attached to a micrometer screw and, after insertion of a second wire, to an isometric force transducer (Kister Morse, DSG 6, Redmond, WA, USA). The preparations were equilibrated for 15 minutes in PSS at 37 °C and the medium was gassed with a mixture of 95% O₂ and 5% CO₂ (pH 7.4). Subsequently the vessels were subjected to a normalization procedure according to Mulvany & Halpern [16]. The individual circumference was adjusted to 90% of the value that the particular vessel would have had at a transmural pressure of 100 mmHg.
Prejunctional and postjunctional AT₁-blockade by eprosartan and candesartan

Electrical field stimulation was applied using thin platinum wire electrodes positioned on either side of the vessel. Contractions were generated using an alternating current of 85/-85 mA with a pulse width of 2 ms for 20 s per frequency step. The frequencies used were 1, 2 and 4 Hz, respectively, applied in succession, in increasing order. Henceforth, the term EFS implies that the full range of frequency steps was applied.

In order to verify whether the stimulation-induced contractions were indeed neuronally mediated, control experiments with tetrodotoxin (1 μM) and prazosin (0.1 μM) were carried out.

Four different types of experiments were performed.

Experiment 1. Ang II + EFS and AT₁-blockade

After an equilibration period of 30 minutes, EFS was applied once as a priming procedure. Another 30 minutes later a second EFS was carried out which served as a reference. After the second EFS (S2) had been carried out, the vessels were left for another 30 minutes. Subsequently, a third EFS (S3) was applied in the presence of angiotensin II (0.5 nM) added to the organ bath 2 minutes before stimulation, or in the presence of the vehicle (control). Finally, a contraction with PSS containing 100 mM KCl (equimolar substitution for NaCl; KPSS) was generated to serve as a reference to quantify the contractions provoked by EFS.

In another group of preparations, multiple concentrations of the AT₁-receptor antagonists were tested. Fifteen minutes after the reference EFS (S2) had been carried out, the medium was replaced by PSS containing one particular concentration of the AT₁-antagonist to be tested. After an incubation period of 15 minutes a third EFS was applied in the presence of the AT₁-antagonist and angiotensin II 0.5 nM (added to the organ bath 2 minutes prior to stimulation).

Experiment 2. Ang II + Noradrenaline

The priming procedure consisted of two subsequent applications of KPSS, a single concentration of phenylephrine (3 μM) and again KPSS. Subsequently, after an equilibration period of 30 minutes, a concentration-response curve (CRC) was constructed for the effects of noradrenaline (concentration range 0.03 – 30 μM) in the presence of either angiotensin II 0.5 nM (added to the organ bath 2 minutes before the CRC was started) or the vehicle (control). Non-linear regression was carried out to calculate maximal effect (Eₘₐₓ) and the concentration NA that caused half-maximal effects (EC₅₀).
Experiment 3. Ang II + EFS and AT$_2$-blockade

The priming procedure was performed as described for experiment 1. After the second EFS (S2) had been carried out, the vessels were left for another 30 minutes. Fifteen minutes after the reference EFS (S2) had been carried out, the medium was replaced by PSS containing the AT$_2$-antagonist PD123319 (10 nM). After an incubation period of 15 minutes a third EFS was applied in the presence of the AT$_2$-antagonist and angiotensin II 0.5 nM (added to the organ bath 2 minutes prior to stimulation).

Experiment 4. Concentration response curves of Ang II and postsynaptic AT$_2$-blockade

The priming procedure was performed as described for experiment 2. Subsequently, after an equilibration period of 30 minutes, a concentration-response curve (CRC) was constructed for the effects of Ang II (concentration range 0.3 nM – 0.3 μM) in the presence of one particular concentration of the AT$_2$-antagonist to be tested (added to the organ bath 15 minutes before the CRC was started) or the vehicle (control). Non-linear regression was carried out to calculate maximal effect ($E_{\text{max}}$) and the concentration Ang II that caused half-maximal effects ($EC_{50}$).

Drugs

Angiotensin II (Bachem, Bubensdorf, Switzerland), (±)-propranolol HCl (Research Biochemical Incorporated, Natick, USA), yohimbine hydrochloride (Sigma Chemical, St. Louis, USA) and N$^\omega$-nitro-L-arginine (Sigma) and PD123319 (Parke Davis, Ann Arbor, USA) was dissolved in distilled water. Eprosartan (Solvay, Hannover, Germany) and candesartan (AstraZeneca, Södertälje, Sweden) were dissolved in NaOH 1 M. Using HCl 1 M, the pH of the solutions was lowered to 7.5. (-)-Noradrenaline bitartrate (Sigma, USA) was dissolved in distilled water containing 1.0(+) ascorbic acid 100 µg/ml.

Statistical analysis

All data are expressed as means ± SEM. Comparison of means was performed using Student's t-test. In case of multiple means, one-way ANOVA and Dunnett's post-test were used. In order to compare the sympatho-inhibitory potency of the AT$_2$-antagonists (stimulation experiments), linear regression was performed and analysis of covariance was used to evaluate differences between regression lines. To compare the potency of the AT$_2$-antagonists regarding inhibition of vasoconstrictor effects of Ang II $pD_2$' values (concentration antagonist that causes 50% reduction of $F_{\text{max}}$) were calculated [17]. A p value < 0.05 was considered to indicate statistical significance.
Prejunctional and postjunctional AT₁-blockade by eprosartan and candesartan

Results

The mean normalized diameter of a total number of 190 mesenteric artery preparations used amounted to 617.5 ± 7.8 μm. Groups consisted of 7-12 animals each. The mean normalized passive tension amounted to 4.5 ± 0.1 mN. The maximum contraction evoked by KPSS amounted to 25.0 ± 0.5 mN. Electrical field stimulation caused an increase of contractile force. This effect could be abolished by tetrodotoxin (1 μM) (N = 4). Prazosin (0.1 μM) abolished the responses to 1 Hz and blocked the responses to 2 and 4 Hz by 92.4 ± 3.0 and 91.3 ± 2.0% (N = 6). These findings demonstrate that the contractions are mainly caused by stimulation-induced release of noradrenaline from the sympathetic nerve terminals.

Responses to S2 (1, 2 and 4 Hz) were 13.8 ± 2.2, 27.6 ± 3.3 and 45.9 ± 3.8% (expressed as % of a standard potassium (100 mM)-contraction). Responses to S3 (1, 2 and 4 Hz) were 14.6 ± 2.3, 26.5 ± 3.5 and 44.9 ± 3.7% (p>0.05 at each frequency). The ratio between forces measured at S2 and S3 (S3/S2) amounted to 1.06 ± 0.03, 0.96 ± 0.03 and 0.98 ± 0.05 for 1, 2 and 4 Hz, respectively. Ang II 0.5 nM caused a significant enhancement of responses to EFS at 1, 2 and 4 Hz. S3/S2-ratios at 1 Hz, 2 Hz and 4 Hz were 2.8 ± 0.5, 2.4 ± 0.4 and 1.6 ± 0.1, respectively (p<0.05 compared to control). The enhancement could be concentration-dependently antagonized by eprosartan (1 nM - 0.1 μM) and candesartan (1 nM - 0.1 μM) (see fig. 1A-B). At the lower concentrations, S-I responses were not significantly different from responses in the presence of Ang II 0.5 nM alone. However, at the higher concentrations responses were significantly weaker, compared to those in the presence of Ang II alone (p<0.05 at all frequencies). In fact, the enhancing effect of Ang II on S-I vasoconstrictor responses was abolished, as reflected by S3 responses which were very similar to S2 responses, resulting in a S3/S2-ratio of approximately 1 at each stimulation frequency. Eprosartan (0.1 μM) and candesartan (0.1 μM) did not cause a change in responses to EFS in the absence of Ang II (N = 4, data not shown).
Figure 1. Inhibitory effect of A: eprosartan (0.01 nM-0.1 μM) and B: candesartan (0.01 nM-0.1 μM) on the facilitation by Ang II of stimulation-induced contractions. Ang II (0.5 nM) in the presence or absence of one of the AT₁-antagonists was added to the organ bath 2 minutes prior to the third electrical field stimulation (S3). The ratio between forces induced by S2 and S3 (S3/S2) is shown at the ordinate and stimulation frequencies at the abscissa. Values are given as means ± SEM. * p<0.05 compared to responses in presence of Ang II (0.5 nM). (N = 7-10 per group).
S-I vasoconstrictor responses as observed at a stimulation frequency of 2 Hz in the presence of Ang II 0.5 nM and the concentrations of eprosartan and candesartan used are plotted in fig. 2. Linear regression adequately described the relationship between S3/S2 in presence of Ang II 0.5 nM and the concentrations of the AT₁-antagonists. R²-values of the regression lines were 0.98 (p<0.05) and 0.97 (p<0.05) for eprosartan and candesartan, respectively. The LR-lines did not differ significantly (P>0.05, analysis of covariance).

Noradrenaline caused a concentration-dependent increase in contractile force. (E<sub>max</sub> 108.5 ± 2.9% of contractions by KPSS, EC<sub>50</sub> 6.08 ± 0.06 -log M). Vasoconstrictor responses to noradrenaline were unaltered by Ang II 0.5 nM (E<sub>max</sub> 114.8 ± 3.1, EC<sub>50</sub> 6.08 ± 0.07, p>0.05 compared to control for both E<sub>max</sub> and EC<sub>50</sub>-values).

**Figure 2.** Inhibitory effects of eprosartan and candesartan on facilitation by Ang II (0.5 nM) of stimulation-induced contractions, observed at a stimulation frequency of 2 Hz. The ratio between forces measured at S2 and S3 (S3/S2) is shown on the ordinate. The concentrations of the AT₁-antagonists, expressed as log M, are shown on the abscissa. Values are given as means ± SEM. Linear regression lines are shown. No statistically significant difference exists between regression lines (p < 0.05, analysis of covariance).
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The AT$_2$-antagonist PD123319 (10 nM) did not have any effect on the Ang II induced facilitation of S-I contractions (fig. 3).

\[ \text{Figure 3. The AT}_2\text{-antagonist PD123319 (10 nM) had no effect on the facilitation by Ang II of stimulation-induced contractions. Ang II (0.5 nM) was added to the organ bath 2 minutes prior to the third electrical field stimulation (S3). The ratio between forces induced by S2 and S3 (S3/S2) is shown at the ordinate and stimulation frequencies at the abscissa. Values are given as means ± SEM. (N = 7 or 10 per group).} \]

Ang II caused a concentration-dependent increase in contractile force. ($E_{\text{max}}$ 63.7 ± 5.0 % of contractions by KPSS, $pEC_{50}$ 8.1 ± 0.09) (Fig. 4). Both eprosartan and candesartan concentration-dependently inhibited contractions to Ang II in a non-competitive manner. $E_{\text{max}}$ was reduced, without causing a rightward-shift of the concentration-response curve to Ang II. In order to compare the potency regarding inhibition of the vasoconstrictor effect of Ang II we calculated $pD_{2}'$-values [17]. $pD_{2}'$-values were 8.8 ± 0.19 and 11.3 ± 0.23 for eprosartan and candesartan respectively (p<0.05). Accordingly, candesartan was considerably more potent than eprosartan regarding the inhibition of the direct vasoconstrictor effect of Ang II.
Prejunctional and postjunctional AT\textsubscript{1}-blockade by eprosartan and candesartan

**Figure 4.** Effects of **A**: eprosartan (0.1 nM – 10 nM) and **B**: candesartan (0.001 nM – 0.1 nM) on the cumulative concentration-response curve for Ang II in the isolated rabbit mesenteric artery. Values are shown as means ± SEM. N = 7-9 per group.
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Discussion

The major findings of the present study are:

1) Ang II, in a subpressor concentration, caused a significant increase in stimulation-induced responses.

2) The enhancement by Ang II of stimulation-induced contractile force could be concentration-dependently antagonized by eprosartan and candesartan. (see fig. 1A-B); eprosartan and candesartan were equipotent regarding this sympatho-inhibitory effect.

3) Ang II (0.5 nM) had no effect on concentration-response curves to exogenous noradrenaline.

4) No effect of the AT2-receptor antagonist PD123319 (10 nM) on facilitation of S-I vasoconstrictor responses by Ang II was observed.

5) Candesartan proved more potent than eprosartan in inhibiting vasoconstrictor effects of angiotensin II.

Our finding that a sub-pressor concentration of Ang II causes such a marked increase of sympathetic neurotransmission, as has been shown in previous studies [11,18,19], demonstrates that this mechanism may well have physiological relevance.

The results of the present study indicate a more important facilitating role of Ang II at the stimulation frequency 1 Hz than at 4 Hz. At 4 Hz, S-I contractions seem to be less sensitive to facilitation by Ang II, probably due to the fact that NA-release is near its maximum at this frequency.

Because we did not observe any effect of Ang II on vasoconstrictor effects of exogenous noradrenaline, we could not confirm an effect of Ang II on α-adrenoceptor mediated responses, reported by other authors in the isolated the rabbit femoral artery [20,21]. Apparently, in the present study, a prejunctional mechanism only is responsibly for the facilitation of Ang II on stimulation-induced responses. In the isolated rabbit ear preparation and coeliac artery, as in the present study, no effect of Ang II on NA-responses was observed [19,22].

The concentration of PD123319 that we used to verify whether the AT2-receptor plays a role in facilitation of sympathetic neurotransmission by Ang II was shown to appropriately and selectively block the AT2-receptor [23]. Hence, our findings are in accordance with other studies, which showed that both in vitro as well as in vivo, the AT2-receptor does not seem to be involved in the facilitation of sympathetic neurotransmission [9].
Prejunctional and postjunctional AT₁-blockade by eprosartan and candesartan

The concentration-response curve to Ang II could be concentration-dependently inhibited by both eprosartan and candesartan (fig. 4). Both compounds caused a depression of the $E_{\text{max}}$, without causing a rightward shift of the concentration-response curve. Accordingly, in this model, both compounds displayed non-competitive antagonism. For candesartan, this has been reported earlier, in the isolated rabbit aorta preparation [24]. However, eprosartan, also in the isolated rabbit aorta, has been reported to be a competitive antagonist, causing a parallel rightward shift of the concentration-response curve, without depression of $E_{\text{max}}$ values [25]. Differences in type of antagonism between different models have been described earlier. We have previously described competitive antagonism for both candesartan and eprosartan in the pithed rat model [15]. Additionally, losartan, which is a competitive antagonist in the rabbit aorta [26], displays mixed antagonism in the rabbit renal artery [27] and non-competitive antagonism in the human forearm [28]. pD$_{2}$' values were $8.8 \pm 0.19$ and $11.3 \pm 0.23$ for candesartan and eprosartan, respectively. Accordingly, in this model, candesartan is a more potent AT$_{1}$-antagonist than eprosartan, as far as the inhibition of direct vasoconstrictor effects of Ang II is concerned.

We compared the potency between the two AT$_{1}$-receptor antagonists in our model both at the pre- and postsynaptic level. We were able to do so because in the same vessel, under carefully standardized conditions it was possible to investigate sympa-tho-inhibition (stimulation-experiments, in the presence and absence of Ang II and AT$_{1}$-blockers) as well as postsynaptic inhibitory potency (cumulative Ang II concentration-response curves in presence and absence of AT$_{1}$-blockers). This approach enabled us to address the issue of relative affinity of the AT$_{1}$-blockers at the presynaptic as well as at the postsynaptic site. Firstly, although regarding sympa-tho-inhibition the two AT$_{1}$-receptor antagonists are equipotent, candesartan appears to be more potent than eprosartan in inhibiting the direct effect of Ang II on the vasculature. In our previous study in the pithed rat, in which we addressed this issue, like in the present investigation, candesartan proved more potent in inhibiting the postsynaptic effects. In contrast with our present findings, however, eprosartan was a more potent inhibitor of the pre-synaptic site [15]. Both the pithed rat data and the present in vitro study indicate that for candesartan, concentrations that inhibit postsynaptic effects of Ang II, sympa-tho-inhibition does not occur. In contrast, for eprosartan, sympa-tho-inhibition occurs at concentrations that inhibit the direct vasoconstrictor effects of Ang II. Our present findings are in accordance with data recently published by Guimarães et al. [29]. In the isolated canine pulmonary artery and rat left ventricle, these authors described sympa-tho-inhibitory properties of eprosartan in concentrations which also block the postsynaptic AT$_{1}$-receptor. In their study, for losartan, sympa-tho-inhibitory concentrations
differed considerably from postsynaptic inhibitory concentrations. In contrast, in isolated atria, Shetty and Delgrande [12] found no significant differences in sympatho-inhibitory potency between eprosartan and losartan.

Our findings may be explained by differences between candesartan and eprosartan in affinity for the presynaptic AT₁-receptor on the one hand and the postsynaptic AT₁-receptor on the other. However, comparison between the two experimental conditions in the present study must be made with utmost care; for the facilitation of sympathetic neurotransmission we used a subpressor concentration of Ang II (0.5 nM) and for direct pressor effects we constructed full concentration-response curves exogenous for Ang II up to concentrations of 0.3 μM.

Taken together, our findings imply that presynaptic AT₁-receptors may consist of a different subtype than postsynaptic receptors. In rodents and rabbits, but not in humans, AT₁A and AT₁B receptor subtypes have been described [23,30]. Differences in subtype between pre- and postsynaptic AT-receptors have been suggested earlier by Guimarães et al. [31]. However, because no radioligand binding studies of AT-receptors on sympathetic nerve terminals are available, this hypothesis remains speculative.

In the present study, inhibition of the pre- and postsynaptically located AT₁-receptor by eprosartan and candesartan were compared. Sympatho-inhibitory properties of AT₁-blockers may be therapeutically relevant, since the sympathetic nervous system has been shown to be involved in the pathogenesis of hypertension and even more so in heart failure [32,33]. Clinical relevance of the findings in the present study remains to be established. Nevertheless, for both of the AT₁-blockers used, the concentrations at which sympatho-inhibition was achieved were similar or even lower than the steady state plasma concentration of the AT₁-blockers used in humans after the usual concentrations [34,35]. Therefore, these findings may well be of clinical relevance.

In conclusion, the present study once more confirms that the facilitating effect of Ang II on the sequelae of neuronal stimulation is mediated by pre-synaptically located AT₁-receptors. Eprosartan is equipotent at pre- and postsynaptic AT₁-receptors, while candesartan is more potent at postjunctional AT₁-receptors compared to the prejunctional ones; in concentrations that inhibit AT₁-receptors mediated vasoconstriction, eprosartan exhibits sympatho-inhibition, whereas candesartan does not.
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