Pre- and postsynaptic studies concerning the interaction between the renin angiotensin system and the sympathetic nervous system
Nap, A.

Citation for published version (APA):
Nap, A. (2003). Pre- and postsynaptic studies concerning the interaction between the renin angiotensin system and the sympathetic nervous system

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CHAPTER 3

Sympatholytic properties of several AT<sub>1</sub>-receptor antagonists in the isolated rabbit thoracic aorta


Alexander Nap, Jippe Balt, Martin Pfaffendorf and Pieter A. van Zwieten
Chapter 3

Introduction

Angiotensin II displays various physiological actions within the cardiovascular system. Vasoconstriction of vascular smooth muscle, secretion of aldosterone by the adrenal cortex, regulation of fluid electrolyte balance and cardiac hypertrophy represent well-known effects mediated by angiotensin II. In addition, several authors have described extensive interactions between angiotensin II and the sympathetic nervous system (SNS) at various levels such as the central nervous system, the adrenal medulla, the sympathetic ganglia and the post-ganglionic nerve terminals. At the nerve terminals angiotensin II has been shown to block noradrenaline uptake, to enhance noradrenaline synthesis and, most importantly, to facilitate sympathetic transmission by enhancement of noradrenaline release. Therefore, at least part of the vasoconstrictor response to angiotensin II appears to involve the facilitation of sympathetic neurotransmission. Hence, the interaction between the SNS and renin-angiotensin-system (RAS) appears relevant, in particular considering the role of the SNS in the genesis and maintenance of cardiovascular diseases such as hypertension and congestive heart failure.

Angiotensin II has been found to stimulate at least two pharmacologically distinct receptors, the AT₁-receptor (agonized by the selective reference compound losartan) and the AT₂-receptor (agonized by PD 123319 and the related agent PD 123177). The major effects of angiotensin II appear to be mediated by the AT₁ receptor subtype, whereas the AT₂ receptor might play an important role in counterbalancing some of the effects mediated by the AT₁ receptor. Whereas in some studies selective AT₁-blockers display no sympatholysis, it is generally assumed that the enhancement of sympathetic neurotransmission is mediated by the AT₂-receptor subtype, based upon in vitro and in vivo studies conducted with losartan, PD 123177 and PD 123319.

In the recent past several in vivo and in vitro studies were conducted in an attempt to compare the potency of various AT₁-antagonists with respect to sympathoinhibition. Eprosartan displayed sympatholytic properties in an in vivo study by Ohlstein et al., whereas several other AT₁-blockers did not. Since the authors assessed merely one dosage of each AT₁-blocker the design of the study is subject to criticism. In contrast, the same blockers, studied in multiple dosages, displayed sympatho-inhibition in two studies by Balt. et al. Significant differences in the sympatholytic potencies were observed, whereas the degree of sympathoinhibition proved equal for all blockers tested.
The majority of these studies, however, consisted of functional experiments in which the sympatholytic properties were evaluated on the basis of the postsynaptic events. Accordingly, the observed effects may have theoretically been influenced by the events within the synaptic cleft and/or at the post-synaptic site. Therefore, to investigate the influence of selective AT₁-antagonists on the exocytotic release the quantification of transmitter is the most straightforward approach. In a previous study we evaluated a modified spillover model that allowed the measurement of sympathetic outflow, using tritium-labeled noradrenaline. This model proved valid to study EFS-evoked sympathetic transmission and its pharmacological modulation, including angiotensin II-mediated enhancement of sympathetic outflow.

Recently, we studied functionally the sympatholytic effect of three AT₁-antagonists in isolated vessels. It seemed interestingly to verify these findings in our aforementioned spillover model, where we evaluated the sympatholytic properties of these selective AT₁-receptor antagonists.

The objective of the present study was to quantify the enhancement of EFS-evoked sympathetic outflow by angiotensin II in rabbit thoracic aortas in order to quantitatively compare the sympatho-inhibitory potency of the selective AT₁-receptor antagonists irbesartan, telmisartan and the reference compound losartan.

Material & Methods

Rabbit isolated thoracic aorta preparations

Experiments were performed with thoracic aortic rings taken from male New Zealand White rabbits, weight 2000-2600g, and obtained from the Common Animal Institute Amsterdam (GDIA). The committee for Animal Experiments of the Academic Medical Center Amsterdam has approved the experimental protocol.

The rabbits were anaesthetized with Hypnorm (fentanyl/fluanison 2.5 mg/kg, i.m.) and sacrificed with Nembutal (pentobarbital 30 mg/kg, i.v.). Before the pentobarbital injection heparin 875IE/kg i.v. was administered. The thoracic cavity was opened and, after removal of the heart and lungs, the thoracic aorta was dissected free from its connective tissue and transferred to a physiological salt solution (PPS), oxygenated by carbogen (95% O₂ + 5% CO₂) and kept at room temperature. Rings of thoracic aorta were prepared and placed in an organ bath set-up.

The medium was composed as follows (mmol/l): NaCl 118, Na₂HPO₄ 1.2, NaHCO₃ 25, KCl 4.7, CaCl₂ 1.6, MgSO₄ 1.2, and glucose 11.0. Ascorbic acid (0.3) and Na₂EDTA (0.03) were added to prevent oxidation of noradrenaline.
Radiolabelling of noradrenergic transmitter stores

In order to label their noradrenergic transmitter stores, the rings were incubated for 45 min in 2.0 ml of PPS containing 0.1 μmol/l l-[^7,8-3H]-noradrenaline (specific activity 28.8 to 52.0 Ci/mmol) in a 5 ml glass-jacketed organ bath. The PPS was continuously bubbled with carbogen and maintained at a temperature of 37°C.

After the incubation period the rings were washed with[^7H]-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. The organ bath contained 20 ml PSS. Desipramine (0.6 μmol/l) and corticosterone (40 μmol/l) were added in order to block uptake-1 and uptake-2 (respectively) of released[^7H]-noradrenaline. Yohimbine (1 μmol/l) was added to the PSS to exclude any α2-adrenergic auto-inhibitory effects of[^7H]-noradrenaline release. The preparations were then 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.

In order to remove superficially bound, non-neuronally released radioactivity the aortic rings were equilibrated for in total 48 min. After an initial period of 18 min the preparations were subjected to a 2 min period of electrical field stimulation (EFS) with a train of 3 ms rectangular bipolar wave pulses of 150mA, at a frequency of 2 Hz ($S_1$) (Danish Myo Technology Current Stimulator, model CS 200). The resulting field strength amounted to 3.57 V/cm (Yokogawa DL 1200; 4 channel 100 MHz). This 'priming' stimulation has proven to increase the reliability and stability of the subsequent basal and EFS-induced[^7H]-noradrenaline spillover.

Stimulation of intrinsic sympathetic nerves

After the equilibration period the aortic preparations were subjected to two periods of electrical field stimulation (trains of 2 min, 3 ms, 150 mA, 2Hz). The first period of stimulation ($S_1$) was applied directly after the equilibration period of 48 min and the tritium outflow thus evoked was taken as control value. Subsequently, a second period ($S_2$) was applied 24 min after $S_1$. The ratio between $S_2$ and $S_1$ was used to quantify the influence exerted by the drugs to be investigated.
Measurement of tritium outflow

Samples of 0.5 ml were repeatedly taken from the organ bath starting at 36 min after washout. Since the organ bath medium was not changed on the one hand and its total volume decreased stepwise by the sampling on the other hand, the actual tritium outflow could be obtained by calculating the incremental accumulation in each sample corrected for the reduced volume. The mean basal tritium efflux/min preceding the stimulation periods $S_1$ and $S_2$ was determined as the mean outflow/min of tritium in two 6 min samples prior to each period of stimulation. For $S_1$, we subtracted the radioactivity (nCi/min) measured in the sample taken at $t=36$ min from that of $t=42$ min and the radioactivity measured in the sample taken at $t=42$ min from that of the sample at $t=48$ min. Hence, we could determine the outflow/min of radioactivity during two 6 min time intervals prior to $S_1$. Basal outflow was determined by averaging these values. An equivalent procedure was used for $S_2$.

The release/min evoked by EFS ($S_1$ and $S_2$, 2 min samples) was calculated by subtracting the corresponding mean basal efflux/min from the apparent EFS-evoked efflux/min.

At the end of the experiment the remaining 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 time at which the EFS was applied or ‘fractional release’ of radioactivity ($FR_1$ and $FR_2$). Accordingly, the effects of pharmacological interventions are expressed as the ratio $FR_2/FR_1$.

Experiment 1: Influence of EFS on $[^3$H]-noradrenaline outflow

In this experiment we quantitatively determined the effects of electrical field stimulation on tritium outflow. EFS ($S_1$) was applied directly after the equilibration period of 48-min and the evoked outflow was compared with the basal tritium outflow. Furthermore, a second stimulation period ($S_2$) was applied in order to quantify the ratio between tritium spillover evoked by consecutive stimulations. The ratio $FR_2/FR_1$ was used to indicate the change in EFS-evoked spillover. To assess whether the EFS-evoked tritium spillover is of neuronal origin we tested the influence of the sodium-channel blocker tetrodotoxin (1 μM). Tetrodotoxin was added twenty minutes before $S_2$.

Experiment 2: Influence of angiotensin II on EFS-evoked sympathetic outflow

To investigate the effect of angiotensin II on sympathetic neurotransmission we tested the influence of angiotensin II (0.01 nM–0.1 μM) on EFS-evoked tritium spillover. Angiotensin II (or the vehicle) was added to the medium, in a non-cumulative manner, two and a half minutes
prior to $S_2$. The ratio $FR_2/FR_1$ was used to indicate the effect of angiotensin II. Each individual preparation was subjected to one concentration of angiotensin II.

**Experiment 3: Influence of selective AT$_1$-receptor antagonists on angiotensin II enhanced EFS-evoked sympathetic outflow**

The selective AT$_1$-receptor antagonists losartan, irbesartan and telmisartan were added in different concentrations to the medium. After an incubation period of twenty minutes a second EFS ($S_2$) was applied in the presence of angiotensin II, in that concentration that maximally enhanced the EFS-evoked tritium spillover (experiment 1). Angiotensin II was added two and a half minutes prior to $S_2$. To indicate the sympatho-inhibitory effects of the different concentrations of the AT$_1$-receptor antagonists the ratio $FR_2/FR_1$ was used.

**Determination of tritium in the samples and tissue**

After the experiment the tissues were kept overnight in 2 ml of 0.5 M quaternary ammonium hydroxide in toluene (Soluene, Packard). Radioactivity was measured by liquid scintillation counting (Tri Carb 2900TR, Packard) in 20-ml aliquots (with either samples or tissue) after the addition of 5 ml of the scintillation mixture (Ultima Gold, Packard). Corrections for counting efficiency were made by external automatic standardization.

**Drugs and chemicals**

Desipramine HCl, yohimbine HCl, tetrodotoxin (Sigma, St. Louis, USA) and angiotensin II (Bachem, Bubendorf, Switzerland, synthetic human sequence) were dissolved in distilled water. Hydrocortisoni hydrogenosuccinas (Bufa, Uitgeest, Holland) was dissolved in DMSO. Stock solutions of desipramine (0.6 mM), yohimbine (1 mM) and corticosterone (40 mM) were further diluted with PPS. Stock solutions of angiotensin II (0.1 mM) were stored in 50-μl aliquots at -20°C.

Irbesartan (Sanofi, Amilly, France) and telmisartan (Boehringer Ingelheim, Germany) were dissolved in NaOH 1M. The pH of these solutions was adjusted to 7.5 using 1M HCl. Losartan (MSD, White House Station, USA) was dissolved in distilled water.

Tritiated levo-[7,8-3H]-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 (Packard, Netherlands).
Statistical analysis
All data are expressed as means ± S.E.M. Student’s *t*-test (two-tailed, unpaired) was used to evaluate statistical significance of differences between means of control and treatment groups. An ANOVA followed by Dunnett’s test was used for multiple comparisons with a control group. Differences at *p*<0.05 were considered to indicate statistical significance.
In order to compare the sympatho-inhibitory potency of the AT₁-antagonists the *fraction of inhibition* was determined by subtracting the individual response from the average response seen in the presence of angiotensin II alone and by dividing that value by the average net increase effected by angiotensin II relative to the average control response (see equation).

\[ \text{Fraction inhibition} = \frac{[\bar{X}(A) - \bar{X}(AB)]}{[\bar{X}(A) - \bar{X}(C)]} \]

A = angiotensin II (1 nM); \( B = \text{AT}_1\)-antagonist; \( C = \text{Control} \)

Linear regression was performed and analysis of covariance was used to evaluate differences between regression lines. Differences at *p*<0.05 were considered to indicate statistical significance. The IC₅₀ values (the concentration AT₁-antagonist that causes 50% reduction in the enhancement of stimulation-induced tritium overflow by angiotensin II) were determined. Differences at *p*<0.05 between the IC₅₀ values were considered to be statistically significant.
Results

**Experiment 1: Influence of EFS on $[^3]$H-noradrenaline outflow**

After a ‘priming’ stimulus rabbit thoracic aortic rings preincubated with $[^3]$H-noradrenaline were subjected to two periods of EFS ($S_1, S_2; 2$Hz, $3$ms, $150$mA, $2$min) at $24$-min intervals. In control experiments, no significant change of the basal efflux of tritium occurred between the periods of stimulation. Stimulation of the aortic rings resulted in nearly a four-fold increase in tritium spillover ($3.32 \pm 0.24$ v.s. $0.79 \pm 0.09$ nCi/min; $n=8$, $p<0.05$) (figure 1). Furthermore, the fractional release of EFS-evoked noradrenaline remained constant throughout the experiment, as shown by the ratio $FR_2/FR_1$ ($0.99 \pm 0.04$, $n=8$).

Tetrodotoxin (1 µM) nearly abolished the EFS-evoked release ($0.06 \pm 0.03$, $n=5$, $p<0.05$), thus confirming that the evoked noradrenaline spillover is indeed of neuronal origin.

![Figure 1](image-url). Mean $[^3]$H-noradrenaline spillover evoked by the first ($S_1$) and the second ($S_2$) period of electrical field stimulation (EFS: $2$Hz, $150$mA, $3$ms, $2$min) in isolated rabbit thoracic aortic rings ($n=8$). Noradrenaline outflow (expressed as nCi/min) is plotted on the ordinate.

**Experiment 2: Influence of angiotensin II on EFS-evoked sympathetic outflow**

Angiotensin II (0.01 nM – 0.1 µM), added to the organ bath 150 seconds before $S_2$, did not alter the resting efflux of tritium (data not shown). In contrast, it caused a concentration-dependent increase of EFS-evoked noradrenaline spillover. The concentration range from 0.1 nM – 0.1 µM angiotensin II resulted in a significant enhancement, by maximally 100% ($FR_2/FR_1$, $2.03 \pm 0.11$), produced by 1 nM angiotensin II ($n=8$). Higher concentrations of angiotensin II evoked less than...
maximal enhancement of the EFS-evoked sympathetic outflow, resulting in a ‘bell-shaped’ curve (figure 2).
Figure 3. Inhibitory effect of losartan (0.1 nM – 0.1 μM) (A), telmisartan (0.01 nM – 10 nM) (B) and irbesartan (0.1 nM – 0.1 μM) (C) on the facilitation by angiotensin II of EFS-activated [3H]-norepinephrine outflow from isolated rabbit thoracic aortic rings. The rings preparations were stimulated at 24-min intervals. Angiotensin II (1 nM) in the presence or absence of the three selective AT1-receptor antagonists was added to the organ bath 150 seconds before S2. The antagonists were added 20 min prior to S2. The ratio between fractional releases evoked by S2 (FR2) and S1 (FR1) is shown in the ordinate and concentrations of the AT1-receptor antagonists (log M) on the abscissa. ■ control; □, angiotensin II; ■, angiotensin II + antagonist. Columns represent the mean ± SEM. Asterisk indicates p<0.05 compared with angiotensin II group (ANOVA followed by Dunnett's test, n=6-7 per group).
The fraction of inhibition displayed by the three lower concentrations of each AT₁-antagonist in the presence of the angiotensin II (1 nM) is shown in figure 4.

A linear correlation was observed between the fraction of inhibition (ordinate) and the concentrations of the AT₁-receptor antagonists (abscissa). The regression line of telmisartan differed significantly from that of irbesartan and losartan. In contrast, no significance was observed between regression lines of irbesartan and losartan.

The IC₅₀ value (that concentration AT₁-antagonist that causes 50% reduction of the enhancement of EFS-evoked spillover by angiotensin II (1 nM)) of telmisartan (pIC₅₀ 10.28 ± 0.20) differed significantly from that of irbesartan (pIC₅₀ 9.20 ± 0.23) and losartan (pIC₅₀ 9.05 ± 0.16) (p<0.05). No statistical significant difference between the IC₅₀ values of irbesartan and losartan was observed.

**Figure 4.** Fraction of inhibition exerted by the lower three concentrations of losartan, telmisartan and irbesartan on the facilitation by angiotensin II (1 nM) of EFS-evoked [³H]-noradrenaline outflow from isolated rabbit thoracic aortic rings. Linear regression analysis adequately described the relationship between fraction of inhibition and concentrations of AT₁-receptor antagonists. Inhibition was determined by subtracting the individual response (of single concentration of antagonist in the presence of angiotensin II) from the average response seen in the presence of angiotensin II alone and by dividing that value by the average net increase effected by angiotensin II relative to the mean basal response (see material and methods). Angiotensin II (1 nM) was added to the organ bath 150 seconds before S₂. The antagonists were added 20 min prior to S₂. The fraction of inhibition is shown on the ordinate and concentrations AT₁-receptor antagonists (log M) on the abscissa. ◆ losartan, □ telmisartan, O irbesartan. Values are given as mean ± SEM. Linear regression lines are shown. Asterisk indicates significant difference between regression lines (p<0.05 analysis of covariance).
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Discussion

In the present study the enhancement of sympathetic neurotransmission by angiotensin II was evaluated. Furthermore, the sympatholytic properties of the selective AT₁-receptor antagonists losartan, telmisartan and irbesartan were determined.

In isolated rabbit thoracic aortic rings, angiotensin II (0.01 nM – 0.1 μM) caused a concentration-dependent enhancement of EFS-evoked sympathetic neurotransmission, with a maximum of approximately 100% (1 nM angiotensin II). Previous studies reported similar results. In vascular and cardiac tissue the observed enhancement of stimulation-induced noradrenaline release by angiotensin II varied from approximately 60% to 90%\textsuperscript{14,15,26}. Further increase of angiotensin II concentrations to 0.1 μM resulted in a less than maximal enhancement. This ‘bell-shaped’ concentration-effect relationship is at general accordance with previous studies\textsuperscript{13,23}, however opposing data has been described\textsuperscript{14}. The nature of such a ‘bell-shaped’ curve is subject of discussion. Several theories may explain this phenomenon, attributing the attenuation of angiotensin II-enhanced sympathetic neurotransmission to concomitant release of prostaglandins\textsuperscript{12,15} or to the existence of a sub-population prejunctionally located AT-receptors that counteract the facilitatory effects mediated by the presynaptic AT₁-receptor\textsuperscript{12}. However, the ‘bell-shaped curve’ does not form the major interest of the present study. Moreover, it is unlikely that in vivo such a phenomenon is present, since rat plasma angiotensin II levels are reported to be significantly lower than the concentrations of angiotensin II used in the current and referred studies\textsuperscript{14}.

To investigate the effect of selective AT₁-receptor antagonists on the angiotensin II-enhanced sympathetic outflow we chose the concentration of angiotensin II (1 nM) that provoked maximal enhancement of [³H]-noradrenaline release.

Losartan (0.1 nM – 0.1 μM), telmisartan (0.01 nM – 10 nM) and irbesartan (0.1 nM – 0.1 μM) concentration-dependently antagonized the enhancement of EFS-evoked sympathetic outflow by angiotensin II (1 nM) (figure 3A-C). We therefore conclude that the presynaptically located AT₁-receptor in the rabbit thoracic aorta belongs to the AT₁-subtype. In addition, the suppression of sympathetic outflow appears to be a class effect of the AT₁-blockers, as already concluded from functional studies\textsuperscript{25}.
To compare the sympatholytic potencies we plotted the fraction of inhibition caused by the three lower concentrations of each antagonist separately (figure 4). A linear correlation was observed between the concentrations applied and the attenuation of angiotensin II-mediated facilitation. Significant differences concerning their relative sympatho-inhibitory potencies were observed, although all AT₁-receptor antagonists attenuated the angiotensin II-induced facilitation equivalently. The pIC₅₀'s indicate that telmisartan is significantly more potent concerning sympatho-inhibition than irbesartan and losartan (p<0.05). The pIC₅₀'s of irbesartan and losartan did not differ significantly. Accordingly, in the isolated rabbit thoracic aorta the order of sympatho-inhibitory potency is telmisartan > irbesartan = losartan.

In general, these findings are at accordance with previous in vitro and in vivo studies. In rat left atria irbesartan was reported to be more potent than losartan with respect to sympatholysis¹⁴. Recently, we observed the order of sympatho-inhibitory potency to be telmisartan > irbesartan > losartan in the rat mesenteric artery²⁸. In the pithed rat, for irbesartan and losartan, the order appears to be reversed, possibly due to the conversion of losartan into its more potent metabolite EXP 3174²⁹. Accordingly, the in vitro order of sympatho-inhibitory potency appears to be telmisartan > irbesartan > losartan. By contrast, we observed no significant difference between irbesartan and losartan concerning sympatho-inhibition. The discrepancy may be explained by species-, tissue- and, for reasons indicated in the introduction, model differences. Nonetheless, variation in the displayed sympatho-inhibitory potency may reveal differences in affinity of the AT₁-receptor blockers for the presynaptic AT₁-receptor.

Whether these results are of clinical relevance in humans remains to be elucidated. However, it can be well imagined that the sympatholytic properties of the AT₁-receptor antagonists may beneficially contribute to their therapeutic effectiveness, since the concentrations of each AT₁-receptor antagonist used in the present study are lower than the steady-state plasma concentrations seen in humans with the typical dosage regimen³⁵-³⁷.

In conclusion, several recent studies emphasized the importance of the assumed sympatholytic properties of clinically used AT₁-receptor antagonists. While most of the studies relied on postjunctional events to quantify prejunctional effects, in the present investigation the exocytotic sympathetic transmitter release was measured directly.

In the present study we used a modified spillover technique to quantify the enhancement of noradrenaline release by angiotensin II and to evaluate the effects of several AT₁-receptor antagonists. This study once more confirms that angiotensin II enhances EFS-evoked
sympathetic transmission, which appears to be mediated by presynaptically located AT₃-receptors. The observed facilitation can be concentration-dependently antagonized by the selective AT₃-receptor antagonists losartan, telmisartan and irbesartan. The order of sympatholytic potency in this model is telmisartan > irbesartan = losartan. These differences might be explained by differences between the individual AT₃-receptor antagonists concerning their affinity for the presynaptic AT₃-receptor.

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