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In vitro and *in vivo* effects of UP 269-6, a new potent orally active nonpeptide angiotensin II receptor antagonist, on vascular smooth muscle cell proliferation

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1 The present studies were designed to measure the affinity of UP 269-6, a newly developed angiotensin AT_1 receptor antagonist, for vascular AT_1 receptors from normotensive and hypertensive rats and to investigate *in vitro*, its effects on angiotensin II (AII)-induced hyperplasia and hypertrophy of vascular smooth muscle cells (VSMC). In addition the *in vivo* effects of UP 269-6 on neointimal proliferation in a carotid artery balloon injury in normotensive rats were also investigated.

2 UP 269-6 selectively inhibited [^{125}I]-Sar^1-Ile^8-AII binding to vascular AT_1 receptors present on VSMC derived from normotensive Wistar rat and from SHR ($K_i = 16.6 \pm 3.6$ nM and 7.5 ± 2.0 nM, respectively). In comparison, losartan and its metabolite, EXP 3174, inhibited [^{125}I]-Sar^1-Ile^8-AII binding to vascular AT_1 receptors derived from both cell models with K_i values slightly lower (losartan) and higher (EXP 3174), respectively, than that of UP 269-6.

3 AII (1 μM) induced a weak and variable hyperplastic response (4 to 32% increase in cell number) in Wistar rat VSMC after 96 h.

4 AII (1 μM) induced a time-dependent increase in cell number in VSMC from SHR. UP 269-6 inhibited concentration-dependently this effect with an IC_{50} value of 159 ± 58 nM. Losartan was clearly less potent and EXP 3174 showed nearly the same inhibitory potency, compared to UP 269-6. UP 269-6 (1 μM) inhibited nearly completely the action of AII.

5 AII (500 nM) caused maximal stimulation of protein synthesis in Wistar rat VSMC (117 ± 36%). UP 269-6, losartan and EXP 3174 totally inhibited this stimulation with IC_{50} values of 28 ± 6 nM, 3504 ± 892 nM and 21 ± 3 nM, respectively.

6 AII (50 nM) induced maximal stimulation of protein synthesis in SHR VSMC (237 ± 67%). UP 269-6, losartan and EXP 3174 totally inhibited this stimulation with IC_{50} values of 16 ± 3 nM, 282 ± 122 nM and 3.3 ± 1.0 nM, respectively.

7 UP 269-6 (75 mg kg^{-1} day^{-1}) administered orally in the diet for 20 days induced a 38% reduction in neointimal area and a 36% reduction in neointima/media ratio associated with the intimal thickening induced by carotid artery balloon injury.

8 In conclusion, UP 269-6 was shown to be a potent antiproliferative agent both *in vitro* on AII-induced hyperplasia and hypertrophy of VSMC derived from normotensive and hypertensive rats, and *in vivo* upon intimal thickening induced by carotid artery balloon injury in the rat.

Keywords: Angiotenisin II receptor; vascular smooth muscle cells (VSMC); [^{125}I]-Sar1-Ile8 binding; normotensive rat VSMC model; hypertensive rat VSMC model; hyperplasia; hypertrophy; neointimal proliferation; UP 269-6; losartan; EXP 3174

Introduction

Excessive proliferation of vascular smooth muscle cells (VSMC) is implicated as a critical event in hypertension, atherosclerosis and restenosis. Several studies suggest that vasopressive hormones may have, in addition to their direct pressor activities, specific mitogenic properties for VSMC *in vitro* and *in vivo*. In particular, there is now considerable evidence implicating a role for the potent vasoconstrictor angiotensin II (AII) in abnormal smooth muscle cell growth. For example, recent studies have demonstrated that treatment of rats with angiotensin-converting enzyme (ACE) inhibitors or AII receptor antagonists significantly decreases myointimal proliferation after acute arterial injury with a balloon catheter (Powell *et al*., 1989, 1991; Prescott *et al*., 1991; Kauffman *et al*., 1991). Furthermore, long-term administration of ACE inhibitors in spontaneously hypertensive rats (SHR) reduces smooth muscle hypertrophy (Owens, 1987).

AII has also been shown to induce hyperplasia and hypertrophy in cultured VSMC. Whilst the hypertrophic action of AII has been confirmed in VSMC from normotensive and hypertensive rat aorta, conflicting results have been obtained regarding its mitogenic effect. In VSMC from SHR, AII exerts a distinct mitogenic action as shown by cell growth and stimulated thymidine incorporation (Paquet *et al*., 1990; Bunkenburg *et al*., 1992). However, in cells derived from normotensive rats, several authors (Geisterfer *et al*., 1988; Berk *et al*., 1989; Paquet *et al*., 1990; Millet *et al*., 1992; Sachinidis *et al*., 1993) have found that AII is not mitogenic *per se*, while others (Weber *et al*., 1994) have shown that AII stimulates normotensive rat VSMC proliferation after 5 days of treatment in the presence of 1% foetal calf serum (FCS).

AII is known to bind to specific high-affinity receptors on the surface of responsive cells and distinct AII receptor genes have been identified (Sasaki *et al*., 1991; Murphy *et al*., 1991; Iwai *et al*., 1991). Vascular AII receptors are believed to be exclusively AT_1 sites.

UP 269-6 is a nonpeptide AT_1 receptor antagonist that has been described to be a potent, orally active, blood pressure-lowering agent in conscious renal hypertensive rats (Nicolaï *et al*., 1994; Caussade *et al*., 1995; Cazes *et al*., 1995).

The aim of the present study was to determine the affinity of UP 269-6 for vascular AT_1 receptors in VSMC derived from normotensive Wistar rat and SHR, then to study its effect on

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AII-induced protein synthesis and cell proliferation in VSMC from both Wistar and SHR. Finally, the *in vivo* effects of UP 269-6 upon neointimal proliferation induced by carotid artery balloon injury in normotensive rats were investigated.

Methods

Isolation and culture of vascular smooth muscle cells

Wistar rat and SHR thoracic aorta were dissected out and smooth muscle cells were isolated by enzymatic digestion by the method described by Bodin *et al.* (1991) with slight modifications. The concentrations of enzymes for media digestion were 87.5 u ml^{-1} of collagenase and 40 u ml^{-1} of elastase (60 u ml^{-1} for one SHR VSMC preparation). This digestion was performed for 1.5 to 2 h at 37°C. The cells were cultured in Dulbecco's modified Eagle medium supplemented with 2 mM glutamine, 100 u ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin and 10% foetal calf serum (DMEM 10% FCS). When cells reached confluence, they exhibited a hill-valley pattern typical for VSMC in culture. At post-confluence, secondary cultures were obtained after harvesting the cells with trypsin 0.1%. Passaged cells were positively identified as smooth muscle cells by immunofluorescence staining for α-actin antibody (1A4 clone) followed by a fluorescein conjugated goat anti-mouse IgG serum. Wistar rat cells from four different preparations at passage levels 5–35 and SHR cells from two different preparations at passage levels 10–21, were used in this study.

[^{125}I]-Sar^{1}-Ile^{8}-angiotensin II binding

Binding studies were carried out on rat cultured aortic smooth muscle cells as previously described (Ullian & Linas, 1990) with slight modifications. VSMC from Wistar rat and SHR were plated in 24-well microplates in Eagle's Minimum Essential Medium (MEM) and Ham's F-10 with Earle's Salts (1:1 vol/vol) supplemented with 2% serum substitutes Ultroser SF/Ultroser G (3:1 vol/vol), 2 mM glutamine, 100 uml^{-1} penicillin, 100 $\mu g\,ml^{-1}$ streptomycin and 0.05 mM ascorbic acid (MEM-HAM 2% Us). At confluence (approximately 5×10^5 cells per well), cells were rinsed with ice-cold incubation buffer (50 mM Tris-HCl, pH 7.2, 100 mM NaCl, 5 mM $MgCl_2$ and 0.25% bovine serum albumin (fraction V, protease free) and incubated in this buffer with 0.2 nM [^{125}I]-Sar^{1}-Ile^{8}-AII and increasing concentrations of the displacing agent. The drugs tested were UP 269-6, losartan and its metabolite, EXP 3174. The total reaction volume was 300 μl. Incubation was performed for 18 h at 4°C. Incubation was terminated by removing the incubation buffer by aspiration and rinsing each well twice with 1 ml of ice-cold washing buffer (50 mM Tris-HCl, pH 7.2, 100 mM NaCl, 5 mM $MgCl_2$). The cells and the bound radioactivity were removed by adding 0.5 ml of lysing buffer (1% sodium dodecyl sulphate, 0.25 N NaOH, pH 10) to each well for at least 15 min. After cell lysis, the solutions were transferred to haemolysis tubes, the wells rinsed once with 0.5 ml of distilled water and the radioactivity counted in a gamma counter. Non specific binding was that remaining in the presence of angiotensin II (10 μM for Wistar rat cells except for experiments with EXP 3174 (5 μM), 0.5 μM for SHR cells except for two experiments (1 μM)) and was found to be about 1–7% of total binding. Each assay was performed in triplicate.

Proliferation studies

Wistar rat or SHR VSMC were seeded at approximately 2×10^4 (Wistar rat) or 3×10^4 (SHR) cells per well into 24-well plates and cultured in DMEM 10% FCS or in MEM-HAM 2% Us, respectively. At 40–50% of confluence, cells were washed with PBS and growth arrested by a 24 h incubation in serum-free DMEM (Wistar rat cells) or MEM-HAM 0.1% Us (SHR cells). Long-term growth was studied after addition of fresh DMEM 0.5% FCS (Wistar rat cells) or MEM-HAM 0.2% Us (SHR cells) supplemented with AII (1 μM). The drugs to be tested (UP 269-6, losartan and EXP 3174) were added one hour before AII. To compare the action of AII with that of classical culture medium, cells were also treated with DMEM 5% or 10% FCS (Wistar rat cells) or MEM-HAM 2% Us (SHR cells). The treatment was renewed every day. Counts were performed every 24 h or only after 72 h (SHR cells) with a Coulter counter, after harvesting by gentle trypsinization. Cell number obtained with DMEM 0.5% FCS (Wistar rat cells) or MEM-HAM 0.2% Us (SHR cells) was taken as control.

Determination of protein synthesis

Wistar rat or SHR VSMC were seeded into 24-well plates at approximately 3×10^4 (Wistar rat) or 4×10^4 (SHR) cells per well and cultured in DMEM 10% FCS and MEM-HAM 2% Us, respectively. At subconfluence, cells were washed with PBS and growth arrested by a 24 or 48 h incubation in serum-free DMEM (Wistar rat cells) or MEM-HAM 0.1% Us (SHR cells).

To study the effects of AII on cell hypertrophy, various concentrations of the peptide were added to the cells in the quiescent medium for 24 h. Following a 20 h incubation period with AII, cells were washed and incubated for one hour in a depletion medium composed of serum-free DMEM without leucine, complemented with glutamine (2 mM) and antibiotics (penicillin 100 u ml^{-1} and streptomycin 100 $\mu g\ ml^{-1}$). AII was added again at this time. [^{3}H]-leucine (1 $\mu Ci\ ml^{-1}$) was added for the last three hours. The incubation medium was then removed, cells were washed once with PBS and the plates were stored at −80°C. After thawing (15 min) and trypsinization, cells were incubated for 30 min at 4°C in 10% trichloroacetic acid (TCA). Thereafter, the TCA-insoluble material was collected on Whatman GF/B glass fibre filters with a cell harvester. Plates were washed three times with distilled water. [^{3}H]-leucine incorporation was determined by liquid scintillation counting. These experiments allowed the determination of the concentration of AII that induced half maximal (EC_{50}) and maximal (EC_{max}) effects.

To study the inhibitory effects of the drugs (UP 269-6, losartan and EXP 3174) on AII-induced hypertrophy, AII (EC_{max}) was added to the cells in the quiescent medium for 24 h. The drugs to be tested were added one hour before AII. The same method as above was then followed.

All the determinations were performed in triplicate. In these experiments, AII (EC_{max}) did not induce any proliferative effect after a 24 h treatment in Wistar rat VSMC as well as in SHR VSMC. Values for quiescent medium-treated cells were taken as 100% and the percentage changes versus these cells were calculated.

Carotid artery injury

The rat left common carotid artery was balloon catheter-injured as described by Clowes *et al.* (1983a). Briefly, male Sprague-Dawley rats weighing 370–390 g were anaesthetized with ketamine 500 (60 mg kg^{-1}, i.p.) and xylazine (8 mg kg^{-1}, i.p.). An arterial embolectomy catheter (2 F Fogarty, Baxter Healthcare Corporation, CA, U.S.A.) was inserted into the left common carotid artery and the intimal injury was accomplished by three passages of the inflated balloon. The external carotid artery was ligated after removal of the catheter and the wound was sutured. In the immediate post-operative periods the animals were wrapped in aluminium foil to prevent hyporthermia. After complete recovery, the animals were housed three to a cage in a temperature (22°C ± 2°C and humidity (55 ± 10%)-controlled room and had free access to rat chow and water. UP 269-6 (12, 30 or 75 mg kg^{-1} day^{-1}) and captopril (100 mg kg^{-1} day^{-1}) were administered orally in the diet for 20 days (6 days before and 14 days after balloon injury). The average daily dose was determined by monitoring food

consumption. Animals given food without compound served as controls. The doses of the two compounds used in this study had previously been determined in separate groups of rats. UP 269-6 (12, 30 or 75 mg kg^{-1} day^{-1} given in the diet for 6 days) produced significant, 4, 9 and 25 fold rightward shifts, respectively, in the AII dose pressor-response curve. Captopril (100 mg kg^{-1} day^{-}) produced a significant 57 fold rightward shift in the AII dose pressor-response curve (data not shown).

Histological measurements were performed two weeks following surgery. Animals were anaesthetized and killed with an overdose of sodium pentobarbitone. The vasculature was rinsed in a retrograde manner through abdominal aorta with Krebs solution for 2–5 min then fixed for 10 min with 3.8% formaldehyde solution by applying a non pulsating physiological level of pressure (100 mm Hg).

Uninjured right and injured left carotid arteries were removed, cleaned of connective tissue and left in Bouin's fluid for 24 h for further fixation. The injured left carotid arteries were divided into five segments (3–4 mm length) and embedded in paraffin. Two cross-section per segment (each 300 μm apart) were cut (5 μm thick) and stained with Verhoeff's elastic stains (Goodfellow *et al*., 1988).

Intimal, medial and lumen cross-sectional areas were quantified by a computerized image analysis system (Samba 2640, Alcatel TITN Answare, Grenoble, France). For each injured carotid artery, morphology parameters were averaged from the analysis of 10 cross sections. A midportion cross-section of uninjured right arteries was used as a normal carotid. Two midportion cross-sections of injured left arteries were stained with fast nuclear red (Gabe, 1968). Cell density within the neointima was determined by automated counting of cells from 2–3 fields of randomly chosen cross-sectional areas representing 20–30% of the total area of an injured vessel. Mean cell area was also determined with a 400 × final magnification on the video screen and after internal calibration of the size of the pixel.

Drugs

[^{125}I]-Sar^{1}-Ile^{8}-angiotensin II (2200 Ci $mmol^{-1}$) was obtained from NEN, Dupont de Nemours (Paris, France) and [^{3}H]-L-leucine (87 Ci $mmol^{-1}$) from Amersham (Les Ulis, France). Angiotensin II, CLS 2 collagenase Worthington, type III elastase, foetal calf serum and α-actin antibody conjugate were purchased from Sigma (supplier: Coger, Paris, France). Goat anti-mouse IgG FITC was from Becton Dickinson (Meylan, France). Cell culture media were supplied from ICN Biomedicals (Orsay, France) and other cell culture reagents from Gibco BRL (Cergy-Pontoise, France). Ascorbic acid was obtained from Sepracor IBF (Villeneuve-la-Garenne, France). Formaldehyde solution (3.8%) was obtained from J.T. Baker (Deventer, Holland). UP 269-6 (5-methyl-7-propyl-8-[2′-(1H-tetrazol-5-yl) biphenyl-4-yl)methyl]-1,2,4-triazolo-[1,5-c] pyrimidin-2(3H)-one) was synthesized by UPSA (Rueil-Malmaison, France). Losartan and EXP 3174 (2-butyl-4-chloro-1-[[2′-(1H-tetrazol-5-yl) biphenyl-4-yl]methyl]imidazole-5-carboxylic acid) were synthesized as samples for pharmacological use by UPSA. Captopril was obtained from Sigma. Ketamine, xylazine and sodium pentobarbitone were obtained from Rhône Mérieux (Lyon, France), Bayer Pharma (Sens, France) and Sanofi Santé animale (Libourne, France), respectively.

Data and statistical analysis

Data are expressed as means ± s.e.mean. Data were analysed by use of Log-logit linear regression programme EBDA (McPherson, 1983; Rodbard, 1984) adapted for an IBM-PC (McPherson, 1985) and obtained from Elsevier-Biosoft (Cambridge, U.K.). Inhibition constant (K_i) values were calculated according to the Cheng-Prusoff equation (Cheng & Prusoff, 1973). The concentrations inducing 50% maximum effect (IC_{50}) were calculated by linear regression. Differences in cell numbers between the treated groups or between treated groups vs control group were tested by analysis of variance. Individual differences were assessed by Dunnett's multiple comparison test. Differences between areas or numbers of cells of treated versus control groups were analyed for statistical significance by unpaired Student's t test. Significance was taken at a value of $P<0.05$.

Results

Binding studies

[^{125}I]-Sar^{1}-Ile^{8}-AII bound to a single population of high affinity binding sites in cultured VSMC from Wistar rats (K_d: 0.49 ± 0.07 nM, B_{max}: 3174 ± 783 fmol mg^{-1} protein, $n=3$) and SHR (K_d: 0.58 ± 0.04 nM; B_{max}: 3261 ± 171 fmol mg^{-1} protein, $n=3$). The specific binding of [^{125}I]-Sar^{1}-Ile^{8}-AII to VSMC from Wistar rat or SHR was displaced in a monophasic manner by UP 269-6, losartan and EXP 3174. K_i values are presented in Table 1.

Proliferation studies

AII (1 μM), in the presence of 0.5% FCS, induced a variable hyperplastic response of Wistar rat VSMC after a 96 h treatment period. The percentage increase in cell number was between 4 and 32% ($n=5$). When AII stimulated cell proliferation occurred, this stimulation was biologically significant since it was inhibited 50% by 0.1 μM UP 269-6.

AII (1 μM) induced a time-dependent increase in VSMC number from SHR (Figure 1), that was significant from 48 h onwards (1.8 fold versus control). Likewise, MEM-HAM 2% Us medium induced a 2.1 fold increase in cell number versus control at 48 h. UP 269-6 (0.1 μM) inhibited AII-induced hyperplasia by 37%, 41% and 39% at 48 h, 72 h and 96 h, respectively. Furthermore, UP 269-6 (1 μM) markedly blocked the mitogenic effect of AII by 83%, 93% and 91% at 48 h, 72 h and 96 h, respectively.

To compare the effects of UP 269-6, losartan and EXP 3174 on AII (1 μM)-induced hyperplasia, cells were treated for 72 h and counted at this time. As shown in Figure 2, the three drugs inhibited the effect of AII in a concentration-dependent manner. UP 269-6 (IC_{50} = 159 ± 58 nM, $n=3$) was 17 fold and 3 fold more potent than losartan (IC_{50} = 2660 ± 601 nM, $n=3$) and EXP 3174 (IC_{50} = 412 ± 92 nM, $n=3$), respectively, in inhibiting AII-induced proliferation of VSMC from SHR.

Hypertrophy studies

AII caused a concentration-dependent increase in leucine incorporation in Wistar rat cells. The EC_{50} value was estimated to be 6.2 ± 0.5 nM ($n=5$) and maximal effects were reached with 500 nM AII. UP 269-6, losartan and EXP 3174 inhibited AII (500 nM)-induced hypertrophy with IC_{50} values of 28 ± 6 nM, 3504 ± 892 nM and 21 ± 3 nM ($n=4$), respectively

Table 1 Inhibition of [^{125}I]-Sar^{1}-Ile^{8}-AII binding to VSMC AT_1 receptors

	Wistar rat K_i (nM)	*SHR* K_i (nM)
UP 269-6	16.6 ± 3.6	7.5 ± 2.0
Losartan	28.5 ± 9.1	27.6 ± 3.4
EXP 3174	3.0 ± 0.4	2.7 ± 0.4

Wistar rat or SHR cells were incubated with [^{125}I]-Sar^{1}-Ile^{8}-AII (0.2 nM) in the presence of various concentrations of UP 269-6, losartan or EXP 3174, for 18 h at 4°C. Specific binding was defined as that remaining in the presence of AII (see text). Values are mean ± s.e.mean from four determinations.

(Figure 3). UP 269-6 was as potent as EXP 3174 and 125 fold more potent than losartan in inhibiting AII-induced hypertrophy.

In SHR cells, AII increased leucine incorporation in a concentration-dependent manner. The EC_{50} value was 3.3 ± 0.8 nM ($n=4$) and maximal effects were reached at 50 nM. UP 269-6, losartan and EXP 3174 inhibited AII (50 nM)-induced hypertrophy with IC_{50} values of 16 ± 3 nM, 282 ± 122 nM and 3.3 ± 1.0 nM ($n=4$), respectively (Figure 4). In SHR cells, UP 269-6 was 18 fold more potent than losartan and 5 fold less potent than EXP 3174 in inhibiting this effect.

Carotid artery injury

The effects of chronic administration of UP 269-6 or captopril upon intimal thickening in response to carotid artery balloon injury are presented in Table 2. In the normal diet-treated group, balloon injury resulted in cross-sectional intimal areas varying from 0.073 to 0.088 mm^2, 14 days post-surgery. UP 269-6 (12 mg kg^{-1} day^{-1}) failed to prevent intimal thickening; the dose of 30 mg kg^{-1} day^{-1} produced a non significant reduction in neointima area (18%) and in neointima/media ratio (10%) as compared to its own control group. However, the higher dose of 75 mg kg^{-1} day^{-1}, UP 269-6 induced a significant reduction in neointimal area (38%) and in neointima/media ratio (36%) as compared to its own control group. Chronic administration of captopril (100 mg kg^{-1} day^{-1}) resulted in a 39% reduction ($P<0.05$) in neointimal area relative to untreated animals. Any reduction in neointima/media ratio (−28%) did not reach a significant level.

UP 269-6 had no significant effect on the area of the media and lumen (Table 2), while captopril significantly decreased the

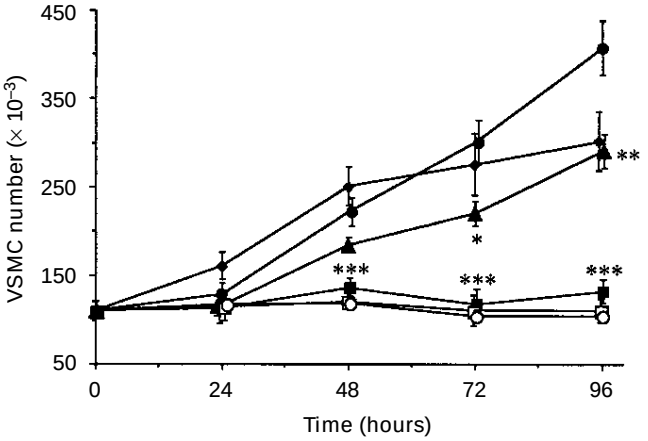

Figure 1 Effect of UP 269-6 on angiotensin II (AII)-induced hyperplasia of VSMC from SHR. SHR VSMC were preincubated in MEM-HAM 0.1% Us for 24 h and treated every 24 h for 96 h with MEM-HAM 0.2% Us as control (○), MEM-HAM 0.2% Us + 1 μM AII alone (●) or in the presence of UP 269-6 0.1 μM (▲) and 1 μM (■); MEM-HAM 0.2% Us + 1 μM UP 269-6 (□) and MEM-HAM 2% Us (◆). Counts were performed every 24 h after cell harvesting by gentle trypsinization. Each point is the mean for triplicate assays in three independent experiments; vertical lines show s.e.mean. AII-induced proliferation was highly significant ($P<0.001$) versus MEM-HAM 0.2% Us from 48 h onwards. Significant difference from MEM-HAM 0.2% Us + 1 μM AII, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

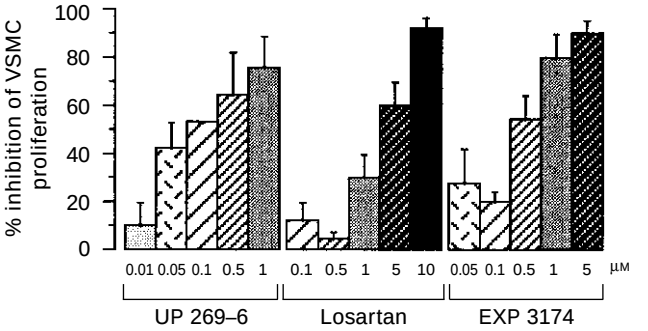

Figure 2 Inhibition by UP 269-6 and other drugs of angiotensin II (AII; 1 μM)-induced hyperplasia in SHR VSMC. SHR VSMC were preincubated in MEM-HAM 0.1% Us for 24 h and treated every 24 h for 72 h with MEM-HAM 0.2% Us and 1 μM AII in the presence of UP 269-6, losartan or EXP 3174. Counts were performed only after 72 h of treatment. Values, expressed as percentage inhibition of AII-induced hyperplasia, are the means ± s.e.mean for triplicate assays in three independent experiments. Drugs exhibited no activity in the absence of AII.

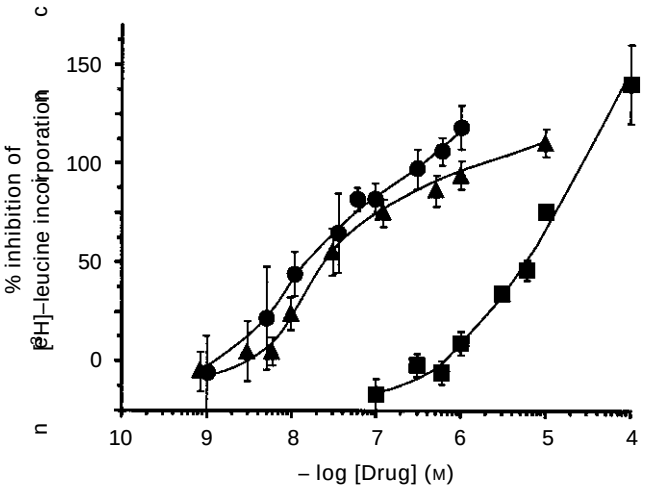

Figure 3 Inhibition of angiotensin II (AII, 500 nM)-induced hypertrophy of VSMC from normotensive Wistar rat. VSMC were preincubated in serum free DMEM for 24 or 48 h and incubated in serum free medium alone, with AII, or with AII and UP 269-6 (▲), losartan (■) or EXP 3174 (●) for 24 h. The drugs were added one hour before AII. Protein synthesis was determined after [^{3}H]-leucine incorporation as described in Methods section. Each data point is the mean of triplicate assays in four independent experiments; vertical lines show s.e.mean.

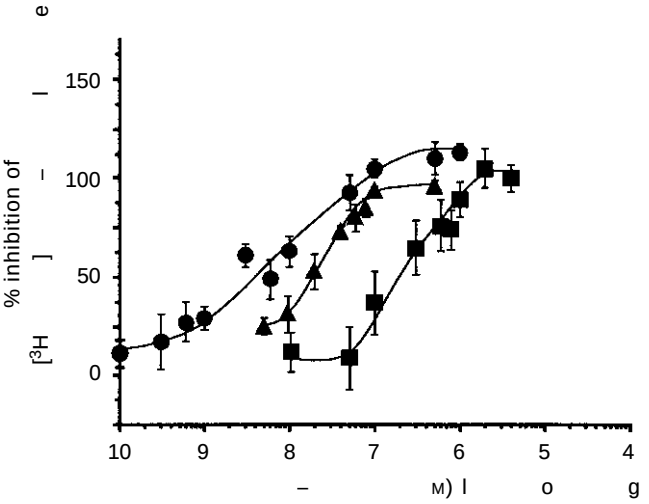

Figure 4 Inhibition of angiotensin II (AII, 50 nM)-induced hypertrophy of VSMC from SHR. VSMC were preincubated in MEM-HAM 0.1% Us for 24 h and incubated in this medium alone, with AII, or with AII and UP 269-6 (▲), losartan (■) or EXP 3174 (●) for 24 h. The drugs were added one hour before AII. Protein synthesis was determined after [^{3}H]-leucine incorporation as described in Methods section. Each data point is the mean of triplicate assays in four independent experiments; vertical lines show s.e.mean.

Table 2 Effect of UP 269-6 and captopril on normotensive rat carotid morphology 14 days after balloon-catheter injury

Experimental group	*Dose*	n	*Neointima area*	*Neointima/media ratio*	*Media area (ballooned)*	*Media area (unballooned)*	*Lumen area (ballooned)*	*Lumen area (unballooned)*
Control		17	8.2±0.6	0.78±0.06	10.7±0.3	8.3±0.7	27.5±1.3	35.7±3.0
UP 269-6	12	13	8.3±0.8	0.78±0.08	10.5±0.4	8.5±1.2	25.6±1.8	38.4±5.2
UP 269-6	30	15	6.7±0.7	0.70±0.07	9.8±0.3	8.6±0.8	26.0±1.4	38.7±3.3
Control		8	7.3±0.8	0.84±0.10	9.0±0.4	9.2±0.7	22.9±2.0	38.9±1.4
UP 269-6	75	16	4.5±0.5*	0.54±0.06*	8.7±0.2	7.9±0.7	23.5±1.4	31.8±3.6
Control		11	8.8±0.9	0.89±0.09	10.0±0.3	11.8±1.6	20.8±1.2	44.1±5.3
Captopril	100	8	5.4±0.9*	0.64±0.12	8.5±0.3**	9.0±1.1	21.1±1.8	33.2±5.4

After staining with Verhoeff's elastic stains, cross-sectional areas were determined with an image analyser (see Methods section for details). Results are given as means±s.e.mean and *n* indicates the number of animals. Significant difference from corresponding control, $*P<0.05$ and $**P<0.01$. Doses and areas are given in mg kg^{-1} day^{-1} and 10^{-2} mm^2, respectively.

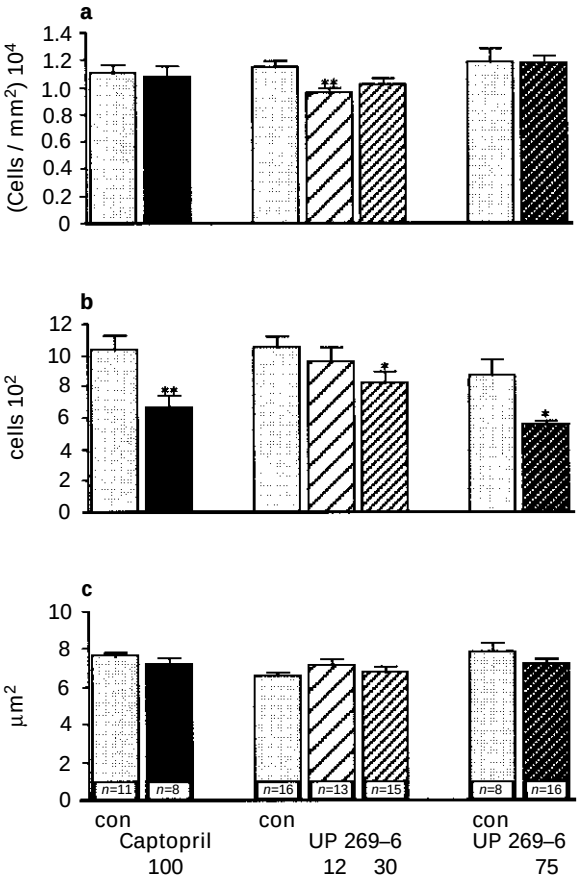

Figure 5 Effect of UP 269-6 and captopril upon cell density (a) total number of cells (b) and mean cell area (c) within the neointima. Two midportion cross-sections of injured arteries were stained with fast nuclear red and cell morphology was assessed with an image analyser (see Methods section for details). Con, control; the numbers refer to the doses given in mg kg^{-1} day^{-1}. Results are given as means± s.e.mean, with the number of rats in each group indicated in the figure. Significant difference from corresponding control, $*P<0.05$ and $**P<0.01$.

medial area (−15%, $P<0.01$) but not the lumen area. Histological analysis of right common carotid arteries (unballooned arteries) revealed no discernible effect of either drug (Table 2).

Cell density within the neointima was not significantly altered by either UP 269-6 (75 mg kg^{-1} day^{-1}) or captopril (100 mg kg^{-1} day^{-1}) (Figure 5a). Surprisingly, UP 269-6 (30 mg kg^{-1} day^{-1}) tended to decrease cell density and a significant reduction (16%) in cell density within the neointima was observed at 12 mg kg^{-1} day^{-1}. When the results were expressed as total number of cells within the neointima, captopril and UP 269-6 (30 and 75 mg kg^{-1} day^{-1}) markedly reduced this parameter as compared to the untreated group (Figure 5b). The total number of cells within the neointima was decreased by UP 269-6 in a dose-dependent manner.

None of the studied compounds had an effect upon mean cell area within the neointima (Figure 5c).

Discussion

The present study was undertaken to determine the *in vitro* effects of UP 269-6, a newly developed AT_1 receptor antagonist, on AII-induced hyperplasia and hypertrophy of VSMC derived from normotensive and hypertensive rats, and its *in vivo* effects on neointimal proliferation in the normotensive rat.

Binding studies

VSMC from adult rat are known to express exclusively the AT_1 receptor subtype, although it has been suggested that these cells have detectable AT_2 binding sites under some culture conditions (Kambayashi *et al*., 1993).

For Wistar rat VSMC, Scatchard analysis revealed a single population of binding sites when [^{125}I]-Sar^1-Ile^8-AII was used as a radioligand, with K_d and B_{max} values comparable with those obtained by Dickinson *et al*. (1994) ($K_d=0.3\pm0.04$ nM and $B_{max}=2860\pm700$ fmol mg^{-1} protein), although with [^{125}I]-AII as radioligand. UP 269-6, as well as losartan and EXP 3174, fully displaced [^{125}I]-Sar^1-Ile^8-AII from the receptors at concentrations demonstrated to be selective for the AT_1 receptor subtype (Caussade *et al*., 1995). The inhibitory potency of UP 269-6 was about 2 fold higher and 5 fold lower than that of losartan and EXP 3174, respectively. The affinity of EXP 3174 was 10 fold higher than that of losartan. Similar differences between losartan and EXP 3174 have also been described by other authors (Chiu *et al*., 1990; Bunkenburg *et al*., 1992; Sacchinidis *et al*., 1993).

Proliferation studies

Our results show that AII can induce a weak and variable hyperplastic response after 96 hours of treatment in Wistar rat quiescent cells cultured in the presence of 0.5% FCS. In some experiments, AII had no effect while, in others, AII induced about a 30% increase in VSMC number. Previous studies of AII-induced growth of smooth muscle cells from normotensive rats have yielded contradictory results. The different rat strains used in these studies may only partially explain such controversy since contradictory results have also been obtained for VSMC derived from the same rat strain. In Sprague-Dawley rat VSMC, AII was found to be a hypertrophic factor only and to have no effect on cell growth (Geisterfer *et al*., 1988; Berk *et al*., 1989) whereas it was found to stimulate DNA synthesis and cell growth by others (Weber *et al*., 1994). AII-induced stimulation of DNA synthesis in VSMC derived from Wistar-Kyoto rats has been

demonstrated (Harris *et al*., 1990; Bunkenburg *et al*., 1992; Koh *et al*., 1994). In our hands, the variations observed in the cellular response to AII seem to be related to cell phenotype changes during cell culture and between different cell preparations.

AII caused a hypertrophic response in Wistar rat VSMC after 24 h, as demonstrated by an increase in protein synthesis. Although it was always observed, the hypertrophic effect was also variable in intensity depending on the cell passage number or cell preparation. These effects of AII on protein synthesis of Wistar rat VSMC were comparable with those already described in different rat strain cells (Koh *et al*., 1954; Berk *et al*., 1989; Bunkenburg *et al*., 1992).

UP 269-6, EXP 3174 and losartan to a lesser degree are antiproliferative agents with respect to the hypertrophic effects of AII in Wistar rat VSMC.

In SHR cells, AII had marked hyperplastic and hypertrophic effects as previously described (Hadrava *et al*., 1989; Bunkenburg *et al*., 1992). AII is a more potent hypertrophic agent on SHR cells than on Wistar rat cells. This increased responsiveness appears not to be due to a difference in the expression of the receptor subtypes between cells derived from the two rat strains. Moreover, it cannot be explained by either a greater receptor denisty in SHR cells or by a higher affinity of the ligands for AT_1 receptors present in SHR-derived cells. A greater receptor density was obtained in VSMC derived from SHR compared with cells from WKY rats (Paquet *et al*., 1990; Bunkenburg *et al*., 1992), but the differences between the two rat strains were slight and variable. We would need more data from further cell preparations to explain our observations.

UP 269-6 was more potent than losartan in inhibiting AII-induced hyperplastic and hypertrophic effects. Surprisingly, when compared with EXP 3174, UP 269-6 was more potent in inhibiting the mitogenic activity of AII (3 fold), but less potent in blocking the hypertrophic activity of AII (5 fold).

Carotid artery injury

It has been shown that captopril markedly prevents neointima formation after balloon catheter-induced vascular injury (Powell *et al*., 1989). Our experimental conditions seem slightly different because balloon injury induced a less intense response than that obtained by those authors (neointima/media ratio was 1.01 to 1.30 in placebo groups). However, the morphometric results confirmed that captopril caused a significant decrease in intimal thickening and had a significant effect on media area.

UP 269-6 also significantly reduced the neointima area and neointima/media ratio at the highest dose tested (75 mg kg^{-1} day^{-1}). This effect corresponded to a marked blockade of vascular AT_1 receptors as assessed by shifts in AII pressor responses (25 fold rightward shift, data not shown). However, even though UP 269-6 produced a 4 and 9 fold rightward shift in the AII dose pressor-response curve, it failed to prevent intimal thickening at 12 and 30 mg kg^{-1} day^{-1}.

Thus, in this model of vascular injury, the effective dose of UP 269-6 for suppressing neointima formation was higher than the dose that inhibits the pressor response to exogenous AII. Similar observations have been described for losartan (Kauffman *et al*., 1991) and cilazapril (Powell *et al*., 1990) and implicate the local renin-angiotensin system in the genesis of this proliferative response.

Neointima formation mainly involves proliferation, migration of VSMC and synthesis of extracellular matrix (Clowes *et al*., 1983b). In the present study UP 269-6 (12 mg kg^{-1} day^{-1}) significantly decreased cell density within the neointima without inhibiting neointima formation, suggesting a probable effect of the drug upon proliferation and/or migration of VSMC without an effect on the amount of extracellular matrix. According to Prescott *et al*. (1991), losartan inhibits both VSMC migration and proliferation. It may be that, at a higher dose which strongly inhibits the pressor response to exogenous AII, a reduction in accumulation of extracellular matrix contributes to the beneficial effect of UP 269-6 on the VSMC. A study on the matrix component needs to be performed (i.e. collagen, fibres, proteoglycan) to test this hypothesis. Finally, neither UP 269-6 nor captopril had an effect on the cross-sectional area of the nucleus of neointimal cells.

In conclusion, the nonpeptide AII receptor antagonist, UP 269-6, is a potent antiproliferative agent active *in vitro* on AII-induced hyperplasia and hypertrophy of VSMC derived from normotensive and hypertensive rat, and *in vivo* upon intimal thickening in response to carotid artery balloon injury. These data confirm the crucial role of AII in the regulation of smooth muscle cell growth and emphasize the great interest and the therapeutic relevance of AII receptor antagonists in vascular disorders.

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